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jc544 U.S. PTO

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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For: IDENTIFICATION OF GENETIC TARGETS FOR MODULATION BY
OLIGONUCLEOTIDES AND GENERATION OF OLIGONUCLEOTIDES FOR
GENE MODULATION

EXPRESS MAIL LABEL NO: EL066387979US

DATE OF DEPOSIT: April 13, 1999

Box ☐ Patent Application
☐ Provisional ☐ Design ☒ Sequence

Assistant Commissioner for Patents
Washington DC 20231

Sir:

PATENT APPLICATION TRANSMITTAL LETTER

Transmitted herewith for filing, please find

☒ A Utility Patent Application under 37 C.F.R. 1.53(b).

It is a continuing application, as follows:

☐ continuation ☐ divisional ☒ continuation-in-part of prior application number
09/067,638, filed April 28, 1998.

- ☐ A Provisional Patent Application under 37 C.F.R. 1.53(c).
- ☐ A Design Patent Application (submitted in duplicate).

Including the following:

- ☐ Provisional Application Cover Sheet.
- ☒ New or Revised Specification, including pages 1 to 131 containing:
- ☒ Specification
 - ☒ Claims
 - ☒ Abstract
 - ☐ Substitute Specification, including Claims and Abstract.
- ☐ The present application is a continuation application of Application No. _____ filed _____. The present application includes the Specification of the parent application which has been revised in accordance with the amendments filed in the parent application. Since none of those amendments incorporate new matter into the parent application, the present revised Specification also does not include new matter.
- ☐ The present application is a continuation application of Application No. _____ filed _____, which in turn is a continuation-in-part of Application No. _____ filed _____. The present application includes the Specification of the parent application which has been revised in accordance with the amendments filed in the parent application. Although the amendments in the parent C-I-P application may have incorporated new matter, since those are the only revisions included in the present application, the present application includes no new matter in relation to the parent application.
- ☐ A copy of earlier application Serial No. _____ Filed _____, including Specification, Claims and Abstract (pages 1 - @@), to which no new matter has been added TOGETHER WITH a copy of the executed oath or declaration for such earlier application and all drawings and appendices. Such earlier application is hereby incorporated into the present application by reference.

- ☐ Please enter the following amendment to the Specification under the Cross-Reference to Related Applications section (or create such a section) : "This Application:
- ☐ is a continuation of ☐ is a divisional of ☐ claims benefit of U.S. provisional Application Serial No. _____ filed _____
- _____
- _____
- _____
- ☐ Signed Statement attached deleting inventor(s) named in the prior application.
- ☐ A Preliminary Amendment.
- ☒ Twenty-four (24) Sheets of ☒ Formal ☐ Informal Drawings.
- ☐ Petition to Accept Photographic Drawings.
- ☐ Petition Fee
- ☒ An ☐ Executed ☒ Unexecuted Declaration or Oath and Power of Attorney.
- ☐ An Associate Power of Attorney.
- ☐ An ☐ Executed ☐ Copy of Executed Assignment of the Invention to _____
- _____
- ☐ A Recordation Form Cover Sheet.
- ☐ Recordation Fee - \$40.00.
- ☐ The prior application is assigned of record to _____
- ☐ Priority is claimed under 35 U.S.C. § 119 of Patent Application No. _____ filed _____ in _____ (country).
- ☐ A Certified Copy of each of the above applications for which priority is claimed:
- ☐ is enclosed.
- ☐ has been filed in prior application Serial No. _____ filed _____
- ☐ An ☐ Executed or ☐ Copy of Executed Earlier Statement Claiming Small Entity

Status under 37 C.F.R. 1.9 and 1.27

☐ is enclosed.

☐ has been filed in prior application Serial No. _____ filed _____, said status is still proper and desired in present case.

☒ Diskette Containing DNA/Amino Acid Sequence Information.

☒ Statement to Support Submission of DNA/Amino Acid Sequence Information.

☐ The computer readable form in this application _____, is identical with that filed in Application Serial Number _____, filed _____. In accordance with 37 CFR 1.821(e), please use the ☐ first-filed, ☐ last-filed or ☐ only computer readable form filed in that application as the computer readable form for the instant application. It is understood that the Patent and Trademark Office will make the necessary change in application number and filing date for the computer readable form that will be used for the instant application. A paper copy of the Sequence Listing is ☐ included in the originally-filed specification of the instant application, ☐ included in a separately filed preliminary amendment for incorporation into the specification.

☐ Information Disclosure Statement.

☐ Attached Form 1449.

☐ Copies of each of the references listed on the attached Form PTO-1449 are enclosed herewith.

☐ A copy of Petition for Extension of Time as filed in the prior case.

☐ Appended Material as follows: _____.

☒ Return Receipt Postcard (should be specifically itemized).

☐ Other as follows: _____

_____.

FEE CALCULATION:

- ☐ Cancel in this application original claims _____ of the prior application before calculating the filing fee. (At least one original independent claim must be retained for filing purposes.)

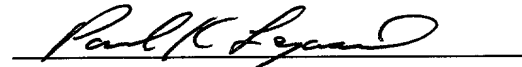
			SMALL ENTITY		NOT SMALL ENTITY	
			RATE	FEE	RATE	FEE
PROVISIONAL APPLICATION			\$75.00	\$	\$150.00	\$
DESIGN APPLICATION			\$155.00	\$	\$310.00	\$
UTILITY APPLICATIONS BASE FEE			\$380.00	\$380.00	\$760.00	\$
UTILITY APPLICATION; ALL CLAIMS CALCULATED AFTER ENTRY OF ALL AMENDMENTS						
	No. Filed	No. Extra				
TOTAL CLAIMS	111 - 20 =	91	\$9 each	\$819.00	\$18 each	\$
INDEP. CLAIMS	45 - 3 =	42	\$39 each	\$1638.00	\$78 each	\$
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM			\$130	\$130	\$260	\$
ADDITIONAL FILING FEE				\$0		\$
TOTAL FILING FEE DUE				\$2967.00		\$

- ☒ A Check is enclosed in the amount of \$ 2967.00.
- ☐ The Commissioner is authorized to charge payment of the following fees and to refund any overpayment associated with this communication or during the pendency of this application to deposit account 23-3050. This sheet is provided in duplicate.
- ☐ The foregoing amount due.
- ☐ Any additional filing fees required, including fees for the presentation of extra claims under 37 C.F.R. 1.16.
- ☐ Any additional patent application processing fees under 37 C.F.R. 1.17 or 1.20(d).
- ☐ The issue fee set in 37 C.F.R. 1.18 at the mailing of the Notice of Allowance.
- ☒ The Commissioner is hereby requested to grant an extension of time for the appropriate length of time, should one be necessary, in connection with this filing or any future filing submitted to the U.S. Patent and Trademark Office in the above-identified application during the pendency of this application. The Commissioner is

further authorized to charge any fees related to any such extension of time to deposit account 23-3050. This sheet is provided in duplicate.

SHOULD ANY DEFICIENCIES APPEAR with respect to this application, including deficiencies in payment of fees, missing parts of the application or otherwise, the United States Patent and Trademark Office is respectfully requested to promptly notify the undersigned.

Date: *April 13, 1999*


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**IDENTIFICATION OF GENETIC TARGETS FOR MODULATION
BY OLIGONUCLEOTIDES AND GENERATION OF
OLIGONUCLEOTIDES FOR GENE MODULATION**

CROSS REFERENCE TO RELATED APPLICATIONS

- 5 The present application is a continuation-in-part of U.S. Serial No. 09/067,638 filed April 28, 1998, which claims priority to provisional application Serial No. 60/081,483 filed April 13, 1998, each of which is incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

- 10 The present invention relates generally to the generation and identification of synthetic compounds having defined physical, chemical or bioactive properties. More particularly, the present invention relates to the automated generation of oligonucleotide compounds targeted to a given nucleic acid sequence via computer-based, iterative robotic synthesis of synthetic oligonucleotide compounds and robotic or robot-assisted analysis of
- 15 the activities of such compounds. Information gathered from assays of such compounds is used to identify nucleic acid sequences that are tractable to a variety of nucleotide sequence-based technologies, for example, antisense drug discovery and target validation.

BACKGROUND OF THE INVENTION

1. Oligonucleotide Technology

Synthetic oligonucleotides of complementarity to targets are known to hybridize with particular, target nucleic acids in a sequence-specific manner. In one example, compounds complementary to the "sense" strand of nucleic acids that encode polypeptides, are referred to as "antisense oligonucleotides." A subset of such compounds may be capable of modulating the expression of a target nucleic acid; such synthetic compounds are described herein as "active oligonucleotide compounds."

Oligonucleotide compounds are commonly used *in vitro* as research reagents and diagnostic aids, and *in vivo* as therapeutic and bioactive agents. Oligonucleotide compounds can exert their effect by a variety of means. One such means takes advantage of an endogenous nuclease, such as RNase H in eukaryotes or RNase P in prokaryotes, to degrade the DNA/RNA hybrid formed between the oligonucleotide sequence and mRNA (Chiang *et al.*, *J. Biol. Chem.*, 1991, 266, 18162; Forster *et al.*, *Science*, 1990, 249, 783). Another means involves covalently linking of a synthetic moiety having nuclease activity to an oligonucleotide having an antisense sequence. This does not rely upon recruitment of an endogenous nuclease to modulate target activity. Synthetic moieties having nuclease activity include, but are not limited to, enzymatic RNAs, lanthanide ion complexes, and other reactive species. (Haseloff *et al.*, *Nature*, 1988, 334, 585; Baker *et al.*, *J. Am. Chem. Soc.*, 1997, 119, 8749).

Despite the advances made in utilizing antisense technology to date, it is still common to identify target sequences amenable to antisense technologies through an empirical approach (Szoka, *Nature Biotechnology*, 1997, 15, 509). Accordingly, the need exists for systems and methods for efficiently and effectively identifying target nucleotide sequences that are suitable for antisense modulation. The present disclosure answers this need by providing systems and methods for automatically identifying such sequences via *in silico*, robotic or other automated means.

2. Identification of Active Oligonucleotide Compounds

Traditionally, new chemical entities with useful properties are generated by (1) identifying a chemical compound (called a "lead compound") with some desirable property or activity, (2) creating variants of the lead compound, and (3) evaluating the

property and activity of such variant compounds. The process has been called "SAR," i.e., structure activity relationship. Although "SAR" and its handmaiden, rational drug design, has been utilized with some degree of success, there are a number of limitations to these approaches to lead compound generation, particularly as it pertains to the discovery of bioactive oligonucleotide compounds. In attempting to use SAR with oligonucleotides, it has been recognized that RNA structure can inhibit duplex formation with antisense compounds, so much so that "moving" the target nucleotide sequence even a few bases can drastically decrease the activity of such compounds (Lima *et al.*, *Biochemistry*, 1992, 31, 12055).

Heretofore, the preferred method of searching for lead antisense compounds has been the manual synthesis and analysis of such compounds. Consequently, a fundamental limitation of the conventional approach is its dependence upon the availability, number and cost of antisense compounds produced by manual, or at best semi-automated, means. Moreover, the assaying of such compounds has traditionally been performed by tedious manual techniques. Thus, the traditional approach to generating active antisense compounds is limited by the relatively high cost and long time required to synthesize and screen a relatively small number of candidate antisense compounds.

Accordingly, the need exists for systems and methods for efficiently and effectively generating new active antisense and other oligonucleotide compounds targeted to specific nucleic acid sequences. The present disclosure answers this need by providing systems and methods for automatically generating and screening active antisense compounds via robotic and other automated means.

3. Gene Function Analysis

Efforts such as the Human Genome Project are making an enormous amount of nucleotide sequence information available in a variety of forms, e.g., genomic sequences, cDNAs, expressed sequence tags (ESTs) and the like. This explosion of information has led one commentator to state that "genome scientists are producing more genes than they can put a function to" (Kahn, *Science*, 1995, 270, 369). Although some approaches to this problem have been suggested, no solution has yet emerged. For example, methods of looking at gene expression in different disease states or stages of development only provide, at best, an association between a gene and a disease or stage of development

(Nowak, *Science*, 1995, 270, 368). Another approach, looking at the proteins encoded by genes, is developing but “this approach is more complex and big obstacles remain” (Kahn, *Science*, 1995, 270, 369). Furthermore, neither of these approaches allows one to directly utilize nucleotide sequence information to perform gene function analysis.

5 In contrast, antisense technology does allow for the direct utilization of nucleotide sequence information for gene function analysis. Once a target nucleic acid sequence has been selected, antisense sequences hybridizable to the sequence can be generated using techniques known in the art. Typically, a large number of candidate antisense oligonucleotides (ASOs) are synthesized having sequences that are more-or-less randomly
10 spaced across the length of the target nucleic acid sequence (e.g., a “gene walk”) and their ability to modulate the expression of the target nucleic acid is assayed. Cells or animals can then be treated with one or more active antisense oligonucleotides, and the resulting effects determined in order to determine the function(s) of the target gene. Although the practicality and value of this empirical approach to determining gene function has been
15 acknowledged in the art, it has also been stated that this approach “is beyond the means of most laboratories and is not feasible when a new gene sequence is identified, but whose function and therapeutic potential are unknown” (Szoka, *Nature Biotechnology*, 1997, 15, 509).

Accordingly, the need exists for systems and methods for efficiently and
20 effectively determining the function of a gene that is uncharacterized except that its nucleotide sequence, or a portion thereof, is known. The present disclosure answers this need by providing systems and methods for automatically generating active antisense compounds to a target nucleotide sequence via robotic means. Such active antisense compounds are contacted with cells, cell-free extracts, tissues or animals capable of
25 expressing the gene of interest and subsequent biochemical or biological parameters are measured. The results are compared to those obtained from a control cell culture, cell-free extract, tissue or animal which has not been contacted with an active antisense compound in order to determine the function of the gene of interest.

4. Target Validation

30 Determining the nucleotide sequence of a gene is no longer an end unto itself; rather, it is “merely a means to an end. The critical next step is to validate the gene and its

[gene] product as a potential drug target" (Glasser, *Genetic Engineering News*, 1997, 17, 1). This process, i.e., confirming that modulation of a gene that is suspected of being involved in a disease or disorder actually results in an effect that is consistent with a causal relationship between the gene and the disease or disorder, is known as target validation.

5 Efforts such as the Human Genome Project are yielding a vast number of complete or partial nucleotide sequences, many of which might correspond to or encode targets useful for new drug discovery efforts. The challenge represented by this plethora of information is how to use such nucleotide sequences to identify and rank valid targets for drug discovery. Antisense technology provides one means by which this might be
10 accomplished; however, the many manual, labor-intensive and costly steps involved in traditional methods of developing active antisense compounds has limited their use in target validation (Szoka, *Nature Biotechnology*, 1997, 15, 509). Nevertheless, the great target specificity that is characteristic of antisense compounds makes them ideal choices for target validation, especially when the functional roles of proteins that are highly related
15 are being investigated (Albert *et al.*, *Trends in Pharm. Sci.*, 1994, 15, 250).

 Accordingly, the need exists for systems and methods for developing compounds efficiently and effectively that modulate a gene, wherein such compounds can be directly developed from nucleotide sequence information. Such compounds are needed to confirm that modulation of a gene that is thought to be involved in a disease or disorder will in fact
20 cause an *in vitro* or *in vivo* effect indicative of the origin, development, spread or growth of the disease or disorder.

 The present disclosure answers this need by providing systems and methods for automatically generating active oligonucleotide and other compounds, especially antisense compounds, to a target nucleotide sequence via robotic or other automated means. Such
25 active compounds are contacted with a cell culture, cell-free extract, tissue or animal capable of expressing the gene of interest, and subsequent biochemical or biological parameters indicative of the potential gene product function are measured. These results are compared to those obtained with a control cell system, cell-free extract, tissue or animal which has not been contacted with an active antisense compound in order to
30 determine whether or not modulation of the gene of interest affects a specific cellular function. The resulting active antisense compounds may be used as positive controls when

other, non antisense-based agents directed to the same target nucleic acid, or to its gene product, are screened.

It should be noted that embodiments of the invention drawn to gene function analysis and target validation have parameters that are shared with other embodiments of the invention, but also have unique parameters. For example, antisense drug discovery naturally requires that the toxicity of the antisense compounds be manageable, whereas, for gene function analysis or target validation, overt toxicity resulting from the antisense compounds is acceptable unless it interferes with the assay being used to evaluate the effects of treatment with such compounds.

U.S. Patent 5,563,036 to Peterson *et al.* describes systems and methods of screening for compounds that inhibit the binding of a transcription factor to a nucleic acid. In a preferred embodiment, an assay portion of the process is stated to be performed by a computer controlled robot.

U.S. Patent 5,708,158 to Hoey describes systems and methods for identifying pharmacological agents stated to be useful for diagnosing or treating a disease associated with a gene the expression of which is modulated by a human nuclear factor of activated T cells. The methods are stated to be particularly suited to high-throughput screening wherein one or more steps of the process are performed by a computer controlled robot.

U.S. Patents 5,693,463 and 5,716,780 to Edwards *et al.* describe systems and methods for identifying non-oligonucleotide molecules that specifically bind to a DNA molecule based on their ability to compete with a DNA-binding protein that recognizes the DNA molecule.

U.S. Patents 5,463,564 and 5,684,711 to Agrafiotis *et al.* describe computer based iterative processes for generating chemical entities with defined physical, chemical and/or bioactive properties.

SUMMARY OF THE INVENTION

The present invention is directed to automated systems and methods for defining sets of compounds that modulate the expression of target nucleic acid sequences, and generating sets of oligonucleotides that modulate the expression of target nucleic acid sequences. The present invention is also directed to identifying nucleic acid sequences

amenable to antisense binding of oligonucleotides to those nucleic acid sequences by the systems and methods of the invention. For purposes of illustration, the present invention is described herein with respect to the production and identification of active antisense oligonucleotides; however, the present invention is not limited to this embodiment.

5 The present invention is directed to iterative processes for defining chemical compounds with prescribed sets of physical, chemical and/or biological properties, and to systems for implementing these processes. During each iteration of a process as contemplated herein, a target nucleic acid sequence is provided or selected, and a library of (candidate) virtual compounds is generated *in silico* (that is in a computer manipulatable
10 and reliable form) according to defined criteria. A library of virtual compounds is generated. These virtual compounds are reviewed and compounds predicted to have particular desired properties are selected. The selected compounds are synthesized, preferably in a robotic, batchwise manner; and then they are robotically assayed for a desired physical, chemical or biological activity in order to identify compounds with the
15 desired properties. Active compounds are, thus, generated and, at the same time, preferred sequences and regions of the target nucleic acid that are amenable to modulation are identified. The preferred compounds of the invention are oligonucleotides that bind to a target nucleic acid sequence.

 In subsequent iterations of the process, second libraries of candidate compounds
20 are generated and/or selected to give rise to a second virtual compound library. Through multiple iterations of the process, a library of target nucleic acid sequences that are tractable to modulation via binding of these compounds to the nucleic acid sequence are identified. Such modulation includes, but is not limited to, antisense technology, gene function analysis and target validation.

25 The present invention is also directed to processes for validating the function of a gene or the product of the gene comprising generating *in silico* a library of nucleobase sequences targeted to the gene and robotically assaying a plurality of synthetic compounds having at least some of the nucleobase sequences for effects on biological function.

 Further features and advantages of the present invention, as well as the structure
30 and operation of various embodiments of the present invention, are described in detail below with reference to the accompanying drawings. In the drawings, like reference

numbers indicate identical or functionally similar elements.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention will be described with reference to the accompanying drawings, wherein:

5 Figures 1 and 2 are a flow diagram of one method according to the present invention depicting the overall flow of data and materials among various elements of the invention.

Figure 3 is a flow diagram depicting the flow of data and materials among elements of step 200 of Figure 1.

10 Figures 4 and 5 are a flow diagram depicting the flow of data and materials among elements of step 300 of Figure 1.

Figure 6 is a flow diagram depicting the flow of data and materials among elements of step 306 of Figure 4.

15 Figure 7 is another flow diagram depicting the flow of data and materials among elements of step 306 of Figure 4.

Figure 8 is a another flow diagram depicting the flow of data and materials among elements of step 306 of Figure 4.

Figure 9 is a flow diagram depicting the flow of data and materials among elements of step 350 of Figure 5.

20 Figures 10 and 11 are flow diagrams depicting a logical analysis of data and materials among elements of step 400 of Figure 1.

Figure 12 is a flow diagram depicting the flow of data and materials among the elements of step 400 of Figure 1.

25 Figures 13 and 14 are flow diagrams depicting the flow of data and materials among elements of step 500 of Figure 1.

Figure 15 is a flow diagram depicting the flow of data and materials among elements of step 600 of Figure 1.

Figure 16 is a flow diagram depicting the flow of data and materials among elements of step 700 of Figure 1.

30 Figure 17 is a flow diagram depicting the flow of data and materials among the

elements of step **1100** of Figure 2.

Figure 18 is a block diagram showing the interconnecting of certain devices utilized in conjunction with a preferred method of the invention;

Figure 19 is a flow diagram showing a representation of data storage in a relational database utilized in conjunction with one method of the invention;

Figure 20 is a flow diagram depicting the flow of data and materials in effecting a preferred embodiment of the invention as set forth in Example 14;

Figure 21 is a flow diagram depicting the flow of data and materials in effecting a preferred embodiment of the invention as set forth in Example 15;

Figure 22 is a flow diagram depicting the flow of data and materials in effecting a preferred embodiment of the invention as set forth in Example 2;

Figure 23 is a pictorial elevation view of a preferred apparatus used to robotically synthesize oligonucleotides; and

Figure 24 is a pictorial plan view of an apparatus used to robotically synthesize oligonucleotides.

DETAILED DESCRIPTION OF THE INVENTION

Certain preferred methods of this invention are now described with reference to the flow diagram of Figures 1 and 2.

1. Target Nucleic Acid Selection. The target selection process, process step **100**, provides a target nucleotide sequence that is used to help guide subsequent steps of the process. It is generally desired to modulate the expression of the target nucleic acid for any of a variety of purposes, such as, e.g., drug discovery, target validation and/or gene function analysis.

One of the primary objectives of the target selection process, step **100**, is to identify molecular targets that represent significant therapeutic opportunities, to provide new and efficacious means of drug discovery and to determine the function of genes that are uncharacterized except for nucleotide sequence. To meet these objectives, genes are classified based upon specific sets of selection criteria.

One such set of selection criteria concerns the quantity and quality of target nucleotide sequence. There must be sufficient target nucleic acid sequence information

available for oligonucleotide design. Moreover, such information must be of sufficient quality to give rise to an acceptable level of confidence in the data to perform the methods described herein. Thus, the data must not contain too many missing or incorrect base entries. In the case of a target sequence that encodes a polypeptide, such errors can often be detected by virtually translating all three reading frames of the sense strand of the target sequence and confirming the presence of a continuous polypeptide sequence having predictable attributes, e.g., encoding a polypeptide of known size, or encoding a polypeptide that is about the same length as a homologous protein. In any event, only a very high frequency of sequence errors will frustrate the methods of the invention; most oligonucleotides to the target sequence will avoid such errors unless such errors occur frequently throughout the entire target sequence.

Another preferred criterion is that appropriate culturable cell lines or other source of reproducible genetic expression should be available. Such cell lines express, or can be induced to express, the gene comprising the target nucleic acid sequence. The oligonucleotide compounds generated by the process of the invention are assayed using such cell lines and, if such assaying is performed robotically, the cell line is preferably tractable to robotic manipulation such as by growth in 96 well plates. Those skilled in the art will recognize that if an appropriate cell line does not exist, it will nevertheless be possible to construct an appropriate cell line. For example, a cell line can be transfected with an expression vector comprising the target gene in order to generate an appropriate cell line for assay purposes.

For gene function analysis, it is possible to operate upon a genetic system having a lack of information regarding, or incomplete characterization of, the biological function(s) of the target nucleic acid or its gene product(s). This is a powerful agent of the invention. A target nucleic acid for gene function analysis might be absolutely uncharacterized, or might be thought to have a function based on minimal data or homology to another gene. By application of the process of the invention to such a target, active compounds that modulate the expression of the gene can be developed and applied to cells. The resulting cellular, biochemical or molecular biological responses are observed, and this information is used by those skilled in the art to elucidate the function of the target gene.

For target validation and drug discovery, another selection criterion is disease

association. Candidate target genes are placed into one of several broad categories of known or deduced disease association. Level 1 Targets are target nucleic acids for which there is a strong correlation with disease. This correlation can come from multiple scientific disciplines including, but not limited to, epidemiology, wherein frequencies of gene abnormalities are associated with disease incidence; molecular biology, wherein gene expression and function are associated with cellular events correlated with a disease; and biochemistry, wherein the *in vitro* activities of a gene product are associated with disease parameters. Because there is a strong therapeutic rationale for focusing on Level 1 Targets, these targets are most preferred for drug discovery and/or target validation.

Level 2 Targets are nucleic acid targets for which the combined epidemiological, molecular biological, and/or biochemical correlation with disease is not so clear as for Level 1. Level 3 Targets are targets for which there is little or no data to directly link the target with a disease process, but there is indirect evidence for such a link, i.e., homology with a Level 1 or Level 2 target nucleic acid sequence or with the gene product thereof. In order not to prejudice the target selection process, and to ensure that the maximum number of nucleic acids actually involved in the causation, potentiation, aggravation, spread, continuance or after-effects of disease states are investigated, it is preferred to examine a balanced mix of Level 1, 2 and 3 target nucleic acids.

In order to carry out drug discovery, experimental systems and reagents shall be available in order for one to evaluate the therapeutic potential of active compounds generated by the process of the invention. Such systems may be operable *in vitro* (e.g., *in vitro* models of cell:cell association) or *in vivo* (e.g., animal models of disease states). It is also desirable, but not obligatory, to have available animal model systems which can be used to evaluate drug pharmacology.

Candidate targets nucleic acids can also classified by biological processes. For example, programmed cell death ("apoptosis") has recently emerged as an important biological process that is perturbed in a wide variety of diseases. Accordingly, nucleic acids that encode factors that play a role in the apoptotic process are identified as candidate targets. Similarly, potential target nucleic acids can be classified as being involved in inflammation, autoimmune disorders, cancer, or other pathological or dysfunctional processes.

Moreover, genes can often be grouped into families based on sequence homology and biological function. Individual family members can act redundantly, or can provide specificity through diversity of interactions with downstream effectors, or through expression being restricted to specific cell types. When one member of a gene family is associated with a disease process then the rationale for targeting other members of the same family is reasonably strong. Therefore, members of such gene families are preferred target nucleic acids to which the methods and systems of the invention may be applied. Indeed, the potent specificity of antisense compounds for different gene family members makes the invention particularly suited for such targets (Albert *et al.*, *Trends Pharm. Sci.*, 1994, 15, 250). Those skilled in the art will recognize that a partial or complete nucleotide sequence of such family members can be obtained using the polymerase chain reaction (PCR) and “universal” primers, i.e., primers designed to be common to all members of a given gene family.

PCR products generated from universal primers can be cloned and sequenced or directly sequenced using techniques known in the art. Thus, although nucleotide sequences from cloned DNAs, or from complementary DNAs (cDNAs) derived from mRNAs, may be used in the process of the invention, there is no requirement that the target nucleotide sequence be isolated from a cloned nucleic acid. Any nucleotide sequence, no matter how determined, of any nucleic acid, isolated or prepared in any fashion, may be used as a target nucleic acid in the process of the invention.

Furthermore, although polypeptide-encoding nucleic acids provide the target nucleotide sequences in one embodiment of the invention, other nucleic acids may be targeted as well. Thus, for example, the nucleotide sequences of structural or enzymatic RNAs may be utilized for drug discovery and/or target validation when such RNAs are associated with a disease state, or for gene function analysis when their biological role is not known.

2. Assembly of Target Nucleotide Sequence. Figure 3 is a block diagram detailing the steps of the target nucleotide sequence assembly process, process step 200 in accordance with one embodiment of the invention. The oligonucleotide design process, process step 300, is facilitated by the availability of accurate target sequence information. Because of limitations of automated genome sequencing technology, gene sequences are

often accumulated in fragments. Further, because individual genes are often being sequenced by independent laboratories using different sequencing strategies, sequence information corresponding to different fragments is often deposited in different databases. The target nucleic acid assembly process take advantage of computerized homology search algorithms and sequence fragment assembly algorithms to search available databases for related sequence information and incorporate available sequence information into the best possible representation of the target nucleic acid molecule, for example a RNA transcript. This representation is then used to design oligonucleotides, process step **300**, which can be tested for biological activity in process step **700**.

In the case of genes directing the synthesis of multiple transcripts, i.e. by alternative splicing, each distinct transcript is a unique target nucleic acid for purposes of step **300**. In one embodiment of the invention, if active compounds specific for a given transcript isoform are desired, the target nucleotide sequence is limited to those sequences that are unique to that transcript isoform. In another embodiment of the invention, if it is desired to modulate two or more transcript isoforms in concert, the target nucleotide sequence is limited to sequences that are shared between the two or more transcripts.

In the case of a polypeptide-encoding nucleic acid, it is generally preferred that full-length cDNA be used in the oligonucleotide design process step **300** (with full-length cDNA being defined as reading from the 5' cap to the poly A tail). Although full-length cDNA is preferred, it is possible to design oligonucleotides using partial sequence information. Therefore it is not necessary for the assembly process to generate a complete cDNA sequence. Further in some cases it may be desirable to design oligonucleotides targeting introns. In this case the process can be used to identify individual introns at process step **220**.

The process can be initiated by entering initial sequence information on a selected molecular target at process step **205**. In the case of a polypeptide-encoding nucleic acid, the full-length cDNA sequence is generally preferred for use in oligonucleotide design strategies at process step **300**. The first step is to determine if the initial sequence information represents the full-length cDNA, decision step **210**. In the case where the full-length cDNA sequence is available the process advances directly to the oligonucleotide design step **300**. When the full-length cDNA sequence is not available, databases are

searched at process step 212 for additional sequence information.

The algorithm preferably used in process steps 212 and 230 is BLAST (Altschul, *et al.*, *J. Mol. Biol.*, **1990**, 215, 403), or “Gapped BLAST” (Altschul *et al.*, *Nucl. Acids Res.*, 1997, 25, 3389). These are database search tools based on sequence homology used to identify related sequences in a sequence database. The BLAST search parameters are set to only identify closely related sequences. Some preferred databases searched by BLAST are a combination of public domain and proprietary databases. The databases, their contents, and sources are listed in Table 1.

Table 1: Database Sources of Target Sequences

10	Database	Contents	Source
	NR	All non-redundant GenBank, EMBL, DDBJ and PDB sequences	National Center for Biotechnology Information at the National Institutes of Health
	Month	All new or revised GenBank, EMBL, DDBJ and PDB sequences released in the last 30 days	National Center for Biotechnology Information at the National Institutes of Health
	Dbest	Non-redundant database of GenBank, EMBL, DDBJ and EST divisions	National Center for Biotechnology Information at the National Institutes of Health
	Dbsts	Non-redundant database of GenBank, EMBL, DDBJ and STS divisions	National Center for Biotechnology Information at the National Institutes of Health
15	Htgs	High throughput genomic sequences	National Center for Biotechnology Information at the National Institutes of Health

When genomic sequence information is available at decision step 215, introns are removed and exons are assembled into continuous sequence representing the cDNA sequence in process step 220. Exon assembly occurs using the Phrap Assembly Program “Phrap” (Copyright University of Washington Genome Center, Seattle, WA).

20 The Phrap algorithm analyzes sets of overlapping sequences and assembles them into one

continuous sequence referred to as a "contig." The resulting contig is preferably used to search databases for additional sequence information at process step 230. When genomic information is not available, the results of process step 212 are analyzed for individual exons at decision step 225. Exons are frequently recorded individually in databases. If multiple complete exons are identified, they are preferably assembled into a contig using Phrap at process step 250. If multiple complete exons are not identified at decision step 225, then sequences can be analyzed for partial sequence information in decision step 228. ESTs identified in the database dbEST are examples of such partial sequence information. If additional partial information is not found, then the process is advanced to process step 230 at decision step 228. If partial sequence information is found in process 212 then that information is advanced to process step 230 via decision step 228.

Process step 230, decision step 240, decision step 260 and process step 250 define a loop designed to extend iteratively the amount of sequence information available for targeting. At the end of each iteration of this loop, the results are analyzed in decision steps 240 and 260. If no new information is found then the process advances at decision step 240 to process step 300. If there is an unexpectedly large amount of sequence information identified, suggesting that the process moved outside the boundary of the gene into repetitive genomic sequence, then the process is preferably cycled back one iteration and that sequence is advanced at decision step 240 to process step 300. If a small amount of new sequence information is identified, then the loop is iterated such as by taking the 100 most 5-prime (5') and 100 most 3-prime (3') bases and iterating them through the BLAST homology search at process step 230. New sequence information is added to the existing contig at process step 250.

3. *In Silico* Generation of a Set of Nucleobase Sequences and Virtual Oligonucleotides.

For the following steps 300 and 400, they may be performed in the order described below, i.e., step 300 before step 400, or, in an alternative embodiment of the invention, step 400 before step 300. In this alternate embodiment, each oligonucleotide chemistry is first assigned to each oligonucleotide sequence. Then, each combination of oligonucleotide chemistry and sequence is evaluated according to the parameters of step 300. This embodiment has the desirable feature of taking into account the effect of

alternative oligonucleotide chemistries on such parameters. For example, substitution of 5-methyl cytosine (5MeC or m5c) for cytosine in an antisense compound may enhance the stability of a duplex formed between that compound and its target nucleic acid. Other oligonucleotide chemistries that enhance oligonucleotide:[target nucleic acid] duplexes are known in the art (see for example, Freier *et al.*, *Nucleic Acids Research*, 1997, 25, 4429). As will be appreciated by those skilled in the art, different oligonucleotide chemistries may be preferred for different target nucleic acids. That is, the optimal oligonucleotide chemistry for binding to a target DNA might be suboptimal for binding to a target RNA having the same nucleotide sequence.

In effecting the process of the invention in the order step 300 before step 400 as seen in Figure 1, from a target nucleic acid sequence assembled at step 200, a list of oligonucleotide sequences is generated as represented in the flowchart shown in Figures 4 and 5. In step 302, the desired oligonucleotide length is chosen. In a preferred embodiment, oligonucleotide length is between from about 8 to about 30, more preferably from about 12 to about 25, nucleotides. In step 304, all possible oligonucleotide sequences of the desired length capable of hybridizing to the target sequence obtained in step 200 are generated. In this step, a series of oligonucleotide sequences are generated, simply by determining the most 5' oligonucleotide possible and "walking" the target sequence in increments of one base until the 3' most oligonucleotide possible is reached.

In step 305, a virtual oligonucleotide chemistry is applied to the nucleobase sequences of step 304 in order to yield a set of virtual oligonucleotides that can be evaluated *in silico*. Default virtual oligonucleotide chemistries include those that are well-characterized in terms of their physical and chemical properties, e.g., 2'-deoxyribonucleic acid having naturally occurring bases (A, T, C and G), unmodified sugar residues and a phosphodiester backbone.

4. *In Silico* Evaluation of Thermodynamic Properties of Virtual Oligonucleotides.

In step 306, a series of thermodynamic, sequence, and homology scores are preferably calculated for each virtual oligonucleotide obtained from step 305.

Thermodynamic properties are calculated as represented in Figure 6. In step 308, the desired thermodynamic properties are selected. As many or as few as desired can be

selected; optionally, none will be selected. The desired properties will typically include step 309, calculation of the free energy of the target structure. If the oligonucleotide is a DNA molecule, then steps 310, 312, and 314 are performed. If the oligonucleotide is an RNA molecule, then steps 311, 313 and 315 are performed. In both cases, these steps
5 correspond to calculation of the free energy of intramolecular oligonucleotide interactions, intermolecular interactions and duplex formation. In addition, a free energy of oligonucleotide-target binding is preferably calculated at step 316.

Other thermodynamic and kinetic properties may be calculated for oligonucleotides as represented at step 317. Such other thermodynamic and kinetic
10 properties may include melting temperatures, association rates, dissociation rates, or any other physical property that may be predictive of oligonucleotide activity.

The free energy of the target structure is defined as the free energy needed to disrupt any secondary structure in the target binding site of the targeted nucleic acid. This region includes any intra-target nucleotide base pairs that need to be disrupted in order for
15 an oligonucleotide to bind to its complementary sequence. The effect of this localized disruption of secondary structure is to provide accessibility by the oligonucleotide. Such structures will include double helices, terminal unpaired and mismatched nucleotides, loops, including hairpin loops, bulge loops, internal loops and multibranch loops (Serra *et al.*, *Methods in Enzymology*, 1995, 259, 242).

20 The intermolecular free energies refer to inherent energy due to the most stable structure formed by two oligonucleotides; such structures include dimer formation. Intermolecular free energies should also be taken into account when, for example, two or more oligonucleotides, of different sequence are to be administered to the same cell in an assay.

25 The intramolecular free energies refer to the energy needed to disrupt the most stable secondary structure within a single oligonucleotide. Such structures include, for example, hairpin loops, bulges and internal loops. The degree of intramolecular base pairing is indicative of the energy needed to disrupt such base pairing.

The free energy of duplex formation is the free energy of denatured
30 oligonucleotide binding to its denatured target sequence. The oligonucleotide-target binding is the total binding involved, and includes the energies involved in opening up

intra- and inter- molecular oligonucleotide structures, opening up target structure, and duplex formation.

The most stable RNA structure is predicted based on nearest neighbor analysis (Xia, T., *et al.*, *Biochemistry*, 1998, 37, 14719-14735; Serra *et al.*, *Methods in Enzymology*, 1995, 259, 242). This analysis is based on the assumption that stability of a given base pair is determined by the adjacent base pairs. For each possible nearest neighbor combination, thermodynamic properties have been determined and are provided. For double helical regions, two additional factors need to be considered, an entropy change required to initiate a helix and an entropy change associated with self-complementary strands only. Thus, the free energy of a duplex can be calculated using the equation:

$$\Delta G^{\circ}_T = \Delta H^{\circ} - T\Delta S^{\circ}$$

where:

ΔG is the free energy of duplex formation,

ΔH is the enthalpy change for each nearest neighbor,

ΔS is the entropy change for each nearest neighbor, and T is temperature.

The ΔH and ΔS for each possible nearest neighbor combination have been experimentally determined. These latter values are often available in published tables. For terminal unpaired and mismatched nucleotides, enthalpy and entropy measurements for each possible nucleotide combination are also available in published tables. Such results are added directly to values determined for duplex formation. For loops, while the available data is not as complete or accurate as for base pairing, one known model determines the free energy of loop formation as the sum of free energy based on loop size, the closing base pair, the interactions between the first mismatch of the loop with the closing base pair, and additional factors including being closed by AU or UA or a first mismatch of GA or UU. Such equations may also be used for oligoribonucleotide-target RNA interactions.

The stability of DNA duplexes is used in the case of intra- or intermolecular oligodeoxyribonucleotide interactions. DNA duplex stability is calculated using similar equations as RNA stability, except experimentally determined values differ between nearest neighbors in DNA and RNA and helix initiation tends to be more favorable in

DNA than in RNA (SantaLucia *et al.*, *Biochemistry*, 1996, 35, 3555).

Additional thermodynamic parameters are used in the case of RNA/DNA hybrid duplexes. This would be the case for an RNA target and oligodeoxynucleotide. Such parameters were determined by Sugimoto *et al.* (*Biochemistry*, 1995, 34, 11211). In addition to values for nearest neighbors, differences were seen for values for enthalpy of helix initiation.

5. *In Silico* Evaluation of Target Accessibility

Target accessibility is believed to be an important consideration in selecting oligonucleotides. Such a target site will possess minimal secondary structure and thus, will require minimal energy to disrupt such structure. In addition, secondary structure in oligonucleotides, whether inter- or intra-molecular, is undesirable due to the energy required to disrupt such structures. Oligonucleotide-target binding is dependent on both these factors. It is desirable to minimize the contributions of secondary structure based on these factors. The other contribution to oligonucleotide-target binding is binding affinity. Favorable binding affinities based on tighter base pairing at the target site is desirable.

Following the calculation of thermodynamic properties ending at step 317, the desired sequence properties to be scored are selected at step 324. As many or as few as desired can be selected; optionally, none will be selected. These properties include the number of strings of four guanosine residues in a row at step 325 or three guanosine in a row at step 326, the length of the longest string of adenosines at step 327, cytidines at step 328 or uridines or thymidines at step 329, the length of the longest string of purines at step 330 or pyrimidine at step 331, the percent composition of adenosine at step 332, cytidine at step 333, guanosine at step 334 or uridines or thymidines at step 335, the percent composition of purines at step 336 or pyrimidines at step 337, the number of CG dinucleotide repeats at step 338, CA dinucleotide repeats at step 339 or UA or TA dinucleotide repeats at step 340. In addition, other sequence properties may be used as found to be relevant and predictive of antisense efficacy, as represented at step 341.

These sequence properties may be important in predicting oligonucleotide activity, or lack thereof. For example, U.S. Patent 5,523,389 discloses oligonucleotides containing stretches of three or four guanosine residues in a row. Oligonucleotides having such sequences may act in a sequence-independent manner. For an antisense approach, such a

mechanism is not usually desired. In addition, high numbers of dinucleotide repeats may be indicative of low complexity regions which may be present in large numbers of unrelated genes. Unequal base composition, for example, 90% adenosine, can also give non-specific effects. From a practical standpoint, it may be desirable to remove

5 oligonucleotides that possess long stretches of other nucleotides due to synthesis considerations. Other sequence properties, either listed above or later found to be of predictive value may be used to select oligonucleotide sequences.

Following step 341, the homology scores to be calculated are selected in step 342. Homology to nucleic acids encoding protein isoforms of the target, as represented at step

10 343, may be desired. For example, oligonucleotides specific for an isoform of protein kinase C can be selected. Also, oligonucleotides can be selected to target multiple isoforms of such genes. Homology to analogous target sequences, as represented at step 344, may also be desired. For example, an oligonucleotide can be selected to a region common to both humans and mice to facilitate testing of the oligonucleotide in both

15 species. Homology to splice variants of the target nucleic acid, as represented at step 345, may be desired. In addition, it may be desirable to determine homology to other sequence variants as necessary, as represented in step 346.

Following step 346, from which scores were obtained in each selected parameter, a desired range is selected to select the most promising oligonucleotides, as represented at

20 step 347. Typically, only several parameters will be used to select oligonucleotide sequences. As structure prediction improves, additional parameters may be used. Once the desired score ranges are chosen, a list of all oligonucleotides having parameters falling within those ranges will be generated, as represented at step 348.

6. Targeting Oligonucleotides to Functional Regions of a Nucleic Acid.

25 It may be desirable to target oligonucleotide sequences to specific functional regions of the target nucleic acid. A decision is made whether to target such regions, as represented in decision step 349. If it is desired to target functional regions then process step 350 occurs as seen in greater detail in Figure 9. If it is not desired then the process proceeds to step 375.

30 In step 350, as seen in Figure 9, the desired functional regions are selected. Such regions include the transcription start site or 5' cap at step 353, the 5' untranslated region

at step 354, the start codon at step 355, the coding region at step 356, the stop codon at step 357, the 3' untranslated region at step 358, 5' splice sites at step 359 or 3' splice sites at step 360, specific exons at step 361 or specific introns at step 362, mRNA stabilization signal at step 363, mRNA destabilization signal at step 364, poly-adenylation signal at step 365, poly-A addition site at step 366, poly-A tail at step 367, or the gene sequence 5' of known pre-mRNA at step 368. In addition, additional functional sites may be selected, as represented at step 369.

Many functional regions are important to the proper processing of the gene and are attractive targets for antisense approaches. For example, the AUG start codon is commonly targeted because it is necessary to initiate translation. In addition, splice sites are thought to be attractive targets because these regions are important for processing of the mRNA. Other known sites may be more accessible because of interactions with protein factors or other regulatory molecules.

After the desired functional regions are selected and determined, then a subset of all previously selected oligonucleotides are selected based on hybridization to only those desired functional regions, as represented by step 370.

7. Uniform Distribution of Oligonucleotides.

Whether or not targeting functional sites is desired, a large number of oligonucleotide sequences may result from the process thus far. In order to reduce the number of oligonucleotide sequences to a manageable number, a decision is made whether to uniformly distribute selected oligonucleotides along the target, as represented in step 375. A uniform distribution of oligonucleotide sequences will aim to provide complete coverage throughout the complete target nucleic acid or the selected functional regions. A computer-based program is used to automate the distribution of sequences, as represented in step 380. Such a program factors in parameters such as length of the target nucleic acid, total number of oligonucleotide sequences desired, oligonucleotide sequences per unit length, number of oligonucleotide sequences per functional region. Manual selection of oligonucleotide sequences is also provided for by step 385. In some cases, it may be desirable to manually select oligonucleotide sequences. For example, it may be useful to determine the effect of small base shifts on activity. Once the desired number of oligonucleotide sequences is obtained either from step 380 or step 385, then these

oligonucleotide sequences are passed onto step 400 of the process, where oligonucleotide chemistries are assigned.

8. Assignment of Actual Oligonucleotide Chemistry.

Once a set of select nucleobase sequences has been generated according to the preceding process and decision steps, actual oligonucleotide chemistry is assigned to the sequences. An "actual oligonucleotide chemistry" or simply "chemistry" is a chemical motif that is common to a particular set of robotically synthesized oligonucleotide compounds. Preferred chemistries include, but are not limited to, oligonucleotides in which every linkage is a phosphorothioate linkage, and chimeric oligonucleotides in which a defined number of 5' and/or 3' terminal residues have a 2'-methoxyethoxy modification.

Chemistries can be assigned to the nucleobase sequences during general procedure step 400 (Figure 1). The logical basis for chemistry assignment is illustrated in Figures 10 and 11 and an iterative routine for stepping through an oligonucleotide nucleoside by nucleoside is illustrated in Figure 12. Chemistry assignment can be effected by assignment directly into a word processing program, via an interactive word processing program or via automated programs and devices. In each of these instances, the output file is selected to be in a format that can serve as an input file to automated synthesis devices.

9. Oligonucleotide Compounds.

In the context of this invention, in reference to oligonucleotides, the term "oligonucleotide" is used to refer to an oligomer or polymer of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) or mimetics thereof. Thus this term includes oligonucleotides composed of naturally-occurring nucleobases, sugars and covalent internucleoside (backbone) linkages as well as oligonucleotides having non-naturally-occurring portions which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms, i.e., phosphodiester linked A, C, G, T and U nucleosides, because of desirable properties such as, for example, enhanced cellular uptake, enhanced affinity for nucleic acid target and increased stability in the presence of nucleases.

The oligonucleotide compounds in accordance with this invention can be of various lengths depending on various parameters, including but not limited to those discussed above in reference to the selection criteria of general procedure 300. For use as

antisense oligonucleotides compounds of the invention preferably are from about 8 to about 30 nucleobases in length (i.e. from about 8 to about 30 linked nucleosides).

Particularly preferred are antisense oligonucleotides comprising from about 12 to about 25 nucleobases. A discussion of antisense oligonucleotides and some desirable modifications
5 can be found in De Mesmaeker *et al.*, *Acc. Chem. Res.*, 1995, 28, 366. Other lengths of oligonucleotides might be selected for non-antisense targeting strategies, for instance using the oligonucleotides as ribozymes. Such ribozymes normally require oligonucleotides of longer length as is known in the art.

A nucleoside is a base-sugar combination. The base portion of the nucleoside is
10 normally a heterocyclic base. The two most common classes of such heterocyclic bases are the purines and the pyrimidines. Nucleotides are nucleosides that further include a phosphate group covalently linked to the sugar portion of the nucleoside. For those nucleosides that include a normal (where normal is defined as being found in RNA and DNA) pentofuranosyl sugar, the phosphate group can be linked to either the 2', 3' or 5'
15 hydroxyl moiety of the sugar. In forming oligonucleotides, the phosphate groups covalently link adjacent nucleosides to one another to form a linear polymeric compound. In turn the respective ends of this linear polymeric structure can be further joined to form a circular structure, however, open linear structures are generally preferred. Within the oligonucleotide structure, the phosphate groups are commonly referred to as forming the
20 internucleoside backbone of the oligonucleotide. The normal linkage or backbone of RNA and DNA is a 3' to 5' phosphodiester linkage.

Specific examples of preferred oligonucleotides useful in this invention include oligonucleotides containing modified backbones or non-natural internucleoside linkages. As defined in this specification, oligonucleotides having modified backbones include those
25 that retain a phosphorus atom in the backbone and those that do not have a phosphorus atom in the backbone. For the purposes of this specification, and as sometimes referenced in the art, modified oligonucleotides that do not have a phosphorus atom in their internucleoside backbone can also be considered to be oligonucleosides.

10. Selection of Oligonucleotide Chemistries.

30 In a general logic scheme as illustrated in Figures 10 and 11, for each nucleoside position, the user or automated device is interrogated first for a base assignment, followed

by a sugar assignment, a linker assignment and finally a conjugate assignment. Thus for each nucleoside, at process step 410 a base is selected. In selecting the base, base chemistry 1 can be selected at process step 412 or one or more alternative bases are selected at process steps 414, 416 and 418. After base selection is effected, the sugar portion of the nucleoside is selected. Thus for each nucleoside, at process step 420 a sugar is selected that together with the select base will complete the nucleoside. In selecting the sugar, sugar chemistry 1 can be selected at process 422 or one or more alternative sugars are selected at process steps 424, 426 and 428. For each two adjacent nucleoside units, at process step 430, the internucleoside linker is selected. The linker chemistry for the internucleoside linker can be linker chemistry 1 selected at process step 432 or one or more alternative internucleoside linker chemistries are selected at process steps 434, 436 and 438.

In addition to the base, sugar and internucleoside linkage, at each nucleoside position, one or more conjugate groups can be attached to the oligonucleotide via attachment to the nucleoside or attachment to the internucleoside linkage. The addition of a conjugate group is integrated at process step 440 and the assignment of the conjugate group is effected at process step 450.

For illustrative purposes in Figures 10 and 11, for each of the bases, the sugars, the internucleoside linkers, or the conjugates, chemistries 1 through n are illustrated. As described in this specification, it is understood that the number of alternate chemistries between chemistry 1 and alternative chemistry n, for each of the bases, the sugars, the internucleoside linkages and the conjugates, is variable and includes, but is not limited to, each of the specific alternative bases, sugar, internucleoside linkers and conjugates identified in this specification as well as equivalents known in the art.

Utilizing the logic as described in conjunction with Figures 10 and 11, chemistry is assigned, as is shown in Figure 12, to the list of oligonucleotides from general procedure 300. In assigning chemistries to the oligonucleotides in this list, a pointer can be set at process step 452 to the first oligonucleotide in the list and at step 453 to the first nucleotide of that first oligonucleotide. The base chemistry is selected at step 410, as described above, the sugar chemistry is selected at step 420, also as described above, followed by selection of the internucleoside linkage at step 430, also as described above.

At decision 440, the process branches depending on whether a conjugate will be added at the current nucleotide position. If a conjugate is desired, the conjugate is selected at step 450, also as described above.

Whether or not a conjugate was added at decision step 440, an inquiry is made at decision step 454. This inquiry asks if the pointer resides at the last nucleotide in the current oligonucleotide. If the result at decision step 454 is "No," the pointer is moved to the next nucleotide in the current oligonucleotide and the loop including steps 410, 420, 430, 440 and 454 is repeated. This loop is reiterated until the result at decision step 454 is "Yes."

When the result at decision step 454 is "Yes," a query is made at decision step 460 concerning the location of the pointer in the list of oligonucleotides. If the pointer is not at the last oligonucleotide of the list, the "No" path of the decision step 460 is followed and the pointer is moved to the first nucleotide of the next oligonucleotide in the list at process step 458. With the pointer set to the next oligonucleotide in the list, the loop that starts at process steps 453 is reiterated. When the result at decision step 460 is "Yes," chemistry has been assigned to all of the nucleotides in the list of oligonucleotides.

11. Description of Oligonucleotide Chemistries.

As is illustrated in Figure 10, for each nucleoside of an oligonucleotide, chemistry selection includes selection of the base forming the nucleoside from a large palette of different base units available. These may be "modified" or "natural" bases (also reference herein as nucleobases) including the natural purine bases adenine (A) and guanine (G), and the natural pyrimidine bases thymine (T), cytosine (C) and uracil (U). They further can include modified nucleobases including other synthetic and natural nucleobases such as 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo uracils and cytosines particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and

3-deazaguanine and 3-deazaadenine. Further nucleobases include those disclosed in United States Patent No. 3,687,808, those disclosed in the *Concise Encyclopedia Of Polymer Science And Engineering*, pages 858-859, Kroschwitz, J.I., ed. John Wiley & Sons, 1990, those disclosed by Englisch *et al.*, *Angewandte Chemie, International Edition*, 1991, 30, 613, and those disclosed by Sanghvi, Y.S., Chapter 15, *Antisense Research and Applications*, pages 289-302, Crooke, S.T. and Lebleu, B., ed., CRC Press, 1993. Certain of these nucleobases are particularly useful for increasing the binding affinity of the oligomeric compounds of the invention. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyl-adenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2°C (Sanghvi, Y.S., Crooke, S.T. and Lebleu, B., eds., *Antisense Research and Applications*, CRC Press, Boca Raton, 1993, pp. 276-278) and are presently preferred for selection as the base. These are particularly useful when combined with a 2'-O-methoxyethyl sugar modifications, described below.

Representative United States patents that teach the preparation of certain of the above noted modified nucleobases as well as other modified nucleobases include, but are not limited to, the above noted U.S. Patent 3,687,808, as well as U.S. Patents 4,845,205; 5,130,302; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121, 5,596,091; 5,614,617; and 5,681,941, each of which is incorporated herein by reference in its entirety. Reference is also made to allowed United States patent application 08/762,488, filed on December 10, 1996, commonly owned with the present application and which is incorporated herein by reference in its entirety.

In selecting the base for any particular nucleoside of an oligonucleotide, consideration is first given to the need of a base for a particular specificity for hybridization to an opposing strand of a particular target. Thus if an "A" base is required, adenine might be selected however other alternative bases that can effect hybridization in a manner mimicking an "A" base such as 2-aminoadenine might be selected should other consideration, e.g., stronger hybridization (relative to hybridization achieved with adenine), be desired.

As is illustrated in Figure 10, for each nucleoside of an oligonucleotide, chemistry selection includes selection of the sugar forming the nucleoside from a large palette of different sugar or sugar surrogate units available. These may be modified sugar groups, for instance sugars containing one or more substituent groups. Preferred substituent groups comprise the following at the 2' position: OH; F; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; or O, S- or N-alkynyl; wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted C₁ to C₁₀ alkyl or C₂ to C₁₀ alkenyl and alkynyl. Particularly preferred are O[(CH₂)_nO]_mCH₃, O(CH₂)_nOCH₃, O(CH₂)_nNH₂, O(CH₂)_nCH₃, O(CH₂)_nONH₂, and O(CH₂)_nON[(CH₂)_nCH₃]₂, where n and m are from 1 to about 10. Other preferred substituent groups comprise one of the following at the 2' position: C₁ to C₁₀ lower alkyl, substituted lower alkyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH₃, OCN, Cl, Br, CN, CF₃, OCF₃, SOCH₃, SO₂CH₃, ONO₂, NO₂, N₃, NH₂, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide, and other substituents having similar properties. A preferred modification includes 2'-methoxyethoxy (2'-O-CH₂CH₂OCH₃, also known as 2'-O-(2-methoxyethyl), 2'-O-methoxyethyl, or 2'-MOE) (Martin *et al.*, *Helv. Chim. Acta*, 1995, 78, 486) i.e., an alkoxyalkoxy group. A further preferred modification includes 2'-dimethylamino oxyethoxy, i.e., a O(CH₂)₂ON(CH₃)₂ group, also known as 2'-DMAOE, as described in co-owned United States patent application Serial Number 09/016,520, filed on January 30, 1998, which is incorporated herein by reference in its entirety.

Other preferred modifications include 2'-methoxy (2'-O-CH₃), 2'-aminopropoxy (2'-OCH₂CH₂CH₂NH₂) and 2'-fluoro (2'-F). Similar modifications may also be made at other positions on the sugar group, particularly the 3' position of the sugar on the 3' terminal nucleotide or in 2'-5' linked oligonucleotides and the 5' position of 5' terminal nucleotide. The nucleosides of the oligonucleotides may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar.

Representative United States patents that teach the preparation of such modified sugars structures include, but are not limited to, U.S. Patents 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811;

5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053 5,639,873; 5,646,265; 5,658,873; 5,670,633; and 5,700,920, certain of which are commonly owned with the present application, each of which is incorporated herein by reference in its entirety, together with allowed United States patent application 08/468,037, filed on June 5, 1995, which is
5 commonly owned with the present application and which is incorporated herein by reference in its entirety.

As is illustrated in Figure 10, for each adjacent pair of nucleosides of an oligonucleotide, chemistry selection includes selection of the internucleoside linkage. These internucleoside linkages are also referred to as linkers, backbones or oligonucleotide
10 backbones. For forming these nucleoside linkages, a palette of different internucleoside linkages or backbones is available. These include modified oligonucleotide backbones, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates and chiral phosphonates, phosphinates,
15 phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, and boranophosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those having inverted polarity wherein the adjacent pairs of nucleoside units are linked 3'-5' to 5'-3' or 2'-5' to 5'-2'. Various salts, mixed salts and free acid forms are also included.

20 Representative United States patents that teach the preparation of the above phosphorus containing linkages include, but are not limited to, U.S. Patents 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,196; 5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677; 5,476,925; 5,519,126; 5,536,821; 5,541,306; 5,550,111; 5,563,253; 5,571,799; 5,587,361; 5,625,050;
25 and 5,697,248, certain of which are commonly owned with this application, each of which is incorporated herein by reference in its entirety.

Preferred internucleoside linkages for oligonucleotides that do not include a phosphorus atom therein, i.e., for oligonucleosides, have backbones that are formed by short chain alkyl or cycloalkyl intersugar linkages, mixed heteroatom and alkyl or
30 cycloalkyl intersugar linkages, or one or more short chain heteroatomic or heterocyclic intersugar linkages. These include those having morpholino linkages (formed in part from

the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide
5 backbones; amide backbones; and others having mixed N, O, S and CH₂ component parts.

Representative United States patents that teach the preparation of the above oligonucleosides include, but are not limited to, U.S. Patents 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,264,562; 5,264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,610,289;
10 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070; 5,663,312; 5,633,360; 5,677,437; and 5,677,439, certain of which are commonly owned with this application, each of which is incorporated herein by reference in its entirety.

In other preferred oligonucleotides, i.e., oligonucleotide mimetics, both the sugar and the intersugar linkage, i.e., the backbone, of the nucleotide units are replaced with
15 novel groups. The base units are maintained for hybridization with an appropriate nucleic acid target compound. One such oligomeric compound, an oligonucleotide mimetic that has been shown to have excellent hybridization properties, is referred to as a peptide nucleic acid (PNA). In PNA compounds, the sugar-phosphate backbone of an oligonucleotide is replaced with an amide-containing backbone, in particular an
20 aminoethylglycine backbone. The nucleobases are retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone. Representative United States patents that teach the preparation of PNA compounds include, but are not limited to, U.S.: 5,539,082; 5,714,331; and 5,719,262, each of which is incorporated
25 herein by reference in its entirety. Further teaching of PNA compounds can be found in Nielsen *et al.*, *Science*, 1991, 254, 1497.

For the internucleoside linkages, the most preferred embodiments of the invention are oligonucleotides with phosphorothioate backbones and oligonucleosides with heteroatom backbones, and in particular -CH₂-NH-O-CH₂-, -CH₂-N(CH₃)-O-CH₂- [known as a methylene (methylimino) or MMI backbone], -CH₂-O-N(CH₃)-CH₂-, -CH₂-N(CH₃)-
30 N(CH₃)-CH₂- and -O-N(CH₃)-CH₂-CH₂- (wherein the native phosphodiester backbone is represented as -O-P-O-CH₂-) of the above referenced U.S. patent 5,489,677, and the amide

backbones of the above referenced U.S. patent 5,602,240. Also preferred are oligonucleotides having morpholino backbone structures of the above-referenced U.S. Patent 5,034,506.

In attaching a conjugate group to one or more nucleosides or internucleoside linkages of an oligonucleotide, various properties of the oligonucleotide are modified. Thus modification of the oligonucleotides of the invention to chemically link one or more moieties or conjugates to the oligonucleotide are intended to enhance the activity, cellular distribution or cellular uptake of the oligonucleotide. Such moieties include but are not limited to lipid moieties such as a cholesterol moiety (Letsinger *et al.*, *Proc. Natl. Acad. Sci. USA*, 1989, 86, 6553), cholic acid (Manoharan *et al.*, *Bioorg. Med. Chem. Lett.*, 1994, 4, 1053), a thioether, e.g., hexyl-S-tritylthiol (Manoharan *et al.*, *Ann. N.Y. Acad. Sci.*, 1992, 660, 306; Manoharan *et al.*, *Bioorg. Med. Chem. Lett.*, 1993, 3, 2765), a thiocholesterol (Oberhauser *et al.*, *Nucl. Acids Res.*, 1992, 20, 533), an aliphatic chain, e.g., dodecandiol or undecyl residues (Saison-Behmoaras *et al.*, *EMBO J.*, 1991, 10, 111; Kabanov *et al.*, *FEBS Lett.*, 1990, 259, 327; Svinarchuk *et al.*, *Biochimie*, 1993, 75, 49), a phospholipid, e.g., di-hexadecyl-*rac*-glycerol or triethylammonium 1,2-di-O-hexadecyl-*rac*-glycero-3-H-phosphonate (Manoharan *et al.*, *Tetrahedron Lett.*, 1995, 36, 3651; Shea *et al.*, *Nucl. Acids Res.*, 1990, 18, 3777), a polyamine or a polyethylene glycol chain (Manoharan *et al.*, *Nucleosides & Nucleotides*, 1995, 14, 969), or adamantane acetic acid (Manoharan *et al.*, *Tetrahedron Lett.*, 1995, 36, 3651), a palmityl moiety (Mishra *et al.*, *Biochim. Biophys. Acta*, 1995, 1264, 229), or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety (Crooke *et al.*, *J. Pharmacol. Exp. Ther.*, 1996, 277, 923).

Representative United States patents that teach the preparation of such oligonucleotide conjugates include, but are not limited to, U.S. Patents 4,828,979; 4,948,882; 5,218,105; 5,525,465; 5,541,313; 5,545,730; 5,552,538; 5,578,717; 5,580,731; 5,580,731; 5,591,584; 5,109,124; 5,118,802; 5,138,045; 5,414,077; 5,486,603; 5,512,439; 5,578,718; 5,608,046; 4,587,044; 4,605,735; 4,667,025; 4,762,779; 4,789,737; 4,824,941; 4,835,263; 4,876,335; 4,904,582; 4,958,013; 5,082,830; 5,112,963; 5,214,136; 5,082,830; 5,112,963; 5,214,136; 5,245,022; 5,254,469; 5,258,506; 5,262,536; 5,272,250; 5,292,873; 5,317,098; 5,371,241; 5,391,723; 5,416,203; 5,451,463; 5,510,475; 5,512,667; 5,514,785; 5,565,552; 5,567,810; 5,574,142; 5,585,481; 5,587,371; 5,595,726; 5,597,696; 5,599,923;

5,599,928 and 5,688,941, certain of which are commonly owned with the present application, and each of which is herein incorporated by reference in its entirety.

12. Chimeric Compounds.

It is not necessary for all positions in a given compound to be uniformly modified. In fact, more than one of the aforementioned modifications may be incorporated in a single compound or even at a single nucleoside within an oligonucleotide. The present invention also includes compounds which are chimeric compounds. "Chimeric" compounds or "chimeras," in the context of this invention, are compounds, particularly oligonucleotides, which contain two or more chemically distinct regions, each made up of at least one monomer unit, i.e., a nucleotide in the case of an oligonucleotide compound. These oligonucleotides typically contain at least one region wherein the oligonucleotide is modified so as to confer upon the oligonucleotide increased resistance to nuclease degradation, increased cellular uptake, and/or increased binding affinity for the target nucleic acid. An additional region of the oligonucleotide may serve as a substrate for enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids.

By way of example, RNase H is a cellular endonuclease which cleaves the RNA strand of an RNA:DNA duplex. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of oligonucleotide inhibition of gene expression. Consequently, comparable results can often be obtained with shorter oligonucleotides when chimeric oligonucleotides are used, compared to phosphorothioate deoxyoligonucleotides hybridizing to the same target region. Cleavage of the RNA target can be routinely detected by gel electrophoresis and, if necessary, associated nucleic acid hybridization techniques known in the art.

Chimeric antisense compounds of the invention may be formed as composite structures representing the union of two or more oligonucleotides, modified oligonucleotides, oligonucleosides and/or oligonucleotide mimetics as described above. Such compounds have also been referred to in the art as "hybrids" or "gapmers". Representative United States patents that teach the preparation of such hybrid structures include, but are not limited to, U.S. Patents 5,013,830; 5,149,797; 5,220,007; 5,256,775; 5,366,878; 5,403,711; 5,491,133; 5,565,350; 5,623,065; 5,652,355; 5,652,356; and 5,700,922, certain of which are commonly owned with the present application and each of

which is incorporated herein by reference in its entirety, together with commonly owned and allowed United States patent application serial number 08/465,880, filed on June 6, 1995, which is incorporated herein by reference in its entirety.

13. Description of Automated Oligonucleotide Synthesis.

5 In the next step of the overall process (illustrated in Figures 1 and 2), oligonucleotides are synthesized on an automated synthesizer. Although many devices may be employed, the synthesizer is preferably a variation of the synthesizer described in United States patents 5,472,672 and 5,529,756, each of which is incorporated herein by reference in its entirety. The synthesizer described in those patents is modified to include
10 movement in along the Y axis in addition to movement along the X axis. As so modified, a 96-well array of compounds can be synthesized by the synthesizer. The synthesizer further includes temperature control and the ability to maintain an inert atmosphere during all phases of synthesis. The reagent array delivery format employs orthogonal X-axis motion of a matrix of reaction vessels and Y-axis motion of an array of reagents. Each
15 reagent has its own dedicated plumbing system to eliminate the possibility of cross-contamination of reagents and line flushing and/or pipette washing. This in combined with a high delivery speed obtained with a reagent mapping system allows for the extremely rapid delivery of reagents. This further allows long and complex reaction sequences to be performed in an efficient and facile manner.

20 The software that operates the synthesizer allows the straightforward programming of the parallel synthesis of a large number of compounds. The software utilizes a general synthetic procedure in the form of a command (.cmd) file, which calls upon certain reagents to be added to certain wells *via* lookup in a sequence (.seq) file. The bottle position, flow rate, and concentration of each reagent is stored in a lookup table (.tab) file.
25 Thus, once any synthetic method has been outlined, a plate of compounds is made by permutating a set of reagents, and writing the resulting output to a text file. The text file is input directly into the synthesizer and used for the synthesis of the plate of compounds. The synthesizer is interfaced with a relational database allowing data output related to the synthesized compounds to be registered in a highly efficient manner.

30 Building of the .seq, .cmd and .tab files is illustrated in Figure 13. Thus as a part of the general oligonucleotide synthesis procedure 500, for each linker chemistry at

process step **502**, a synthesis file, i.e., a .cmd file, is built at process step **504**. This file can be built fresh to reflect a completely new set of machine commands reflecting a set of chemical synthesis steps or it can modify an existing file stored at process step **504** by editing that stored file in process step **508**. The .cmd files are built using a word processor and a command set of instructions as outlined below.

It will be appreciated that the preparation of control software and data files is within the routine skill of persons skilled in annotated nucleotide synthesis. The same will depend upon the hardware employed, the chemistries adopted and the design paradigm selected by the operator.

In a like manner to the building the .cmd files, .tab files are built to reflect the necessary reagents used in the automatic synthesizer for the particular chemistries that have been selected for the linkages, bases, sugars and conjugate chemistries. Thus for each of a set of these chemistries at process step **510**, a .tab file is built at process step **512** and stored at process step **514**. As with the .cmd files, an existing .tab file can be edited at process step **516**.

Both the .cmd files and the .tab files are linked together at process step **518** and stored for later retrieval in an appropriate sample database **520**. Linking can be as simple as using like file names to associate a .cmd file to its appropriate .tab file, e.g., synthesis_1.cmd is linked to synthesis_1.tab by use of the same preamble in their names.

The automated, multi-well parallel array synthesizer employs a reagent array delivery format, in which each reagent utilized has a dedicated plumbing system. As seen in Figures 23 and 24, an inert atmosphere **522** is maintained during all phases of a synthesis. Temperature is controlled *via* a thermal transfer plate **524**, which holds an injection molded reaction block **526**. The reaction plate assembly slides in the X-axis direction, while for example eight nozzle blocks (**528**, **530**, **532**, **534**, **536**, **538**, **540** and **542**) holding the reagent lines slide in the Y-axis direction, allowing for the extremely rapid delivery of any of 64 reagents to 96 wells. In addition, there are for example, six banks of fixed nozzle blocks (**544**, **546**, **548**, **550**, **552** and **554**) which deliver the same reagent or solvent to eight wells at once, for a total of 72 possible reagents.

In synthesizing oligonucleotides for screening, the target reaction vessels, a 96 well plate **556** (a 2-dimensional array), moves in one direction along the X axis, while the

series of independently controlled reagent delivery nozzles (528, 530, 532, 534, 536, 538, 540 and 542) move along the Y-axis relative to the reaction vessel 558. As the reaction plate 556 and reagent nozzles (528, 530, 532, 534, 536, 538, 540 and 542) can be moved independently at the same time, this arrangement facilitates the extremely rapid delivery of up to 72 reagents independently to each of the 96 reaction vessel wells.

The system software allows the straightforward programming of the synthesis of a large number of compounds by supplying the general synthetic procedure in the form of the command file to call upon certain reagents to be added to specific wells *via* lookup in the sequence file with the bottle position, flow rate, and concentration of each reagent being stored in the separate reagent table file. Compounds can be synthesized on various scales. For oligonucleotides, a 200 nmole scale is typically selected while for other compounds larger scales, as for example a 10 μ mole scale (3-5 mg), might be utilized. The resulting crude compounds are generally >80% pure, and are utilized directly for high throughput screening assays. Alternatively, prior to use the plates can be subjected to quality control (see general procedure 600 and Example 9) to ascertain their exact purity. Use of the synthesizer results in a very efficient means for the parallel synthesis of compounds for screening.

The software inputs accept tab delimited text files (as discussed above for file 504 and 512) from any text editor. A typical command file, a .cmd file, is shown in Example 3 at Table 2. Typical sequence files, .seq files, are shown in Example 3 at Tables 3 and 4 (.SEQ file), and a typical reagent file, a .tab file, is shown in Example 3 at Table 5. Table 3 illustrates the sequence file for an oligonucleotide having 2'-deoxy nucleotides at each position with a phosphorothioate backbone throughout. Table 4 illustrates the sequence file for an oligonucleotide, again having a phosphorothioate backbone throughout, however, certain modified nucleoside are utilized in portions of the oligonucleotide. As shown in this table, 2'-O-(2-methoxyethyl) modified nucleosides are utilized in a first region (a wing) of the oligonucleotide, followed by a second region (a gap) of 2'-deoxy nucleotides and finally a third region (a further wing) that has the same chemistry as the first region. Typically some of the wells of the 96 well plate 556 may be left empty (depending on the number of oligonucleotides to be made during an individual synthesis) or some of the wells may have oligonucleotides that will serve as standards for comparison

or analytical purposes.

Prior to loading reagents, moisture sensitive reagent lines are purged with argon at **522** for 20 minutes. Reagents are dissolved to appropriate concentrations and installed on the synthesizer. Large bottles, collectively identified as **558** in Figure 23 (containing 8
5 delivery lines) are used for wash solvents and the delivery of general activators, trityl group cleaving reagents and other reagents that may be used in multiple wells during any particular synthesis. Small septa bottles, collectively identified as **560** in Figure 23, are utilized to contain individual nucleotide amidite precursor compounds. This allows for anhydrous preparation and efficient installation of multiple reagents by using needles to
10 pressurize the bottle, and as a delivery path. After all reagents are installed, the lines are primed with reagent, flow rates measured, then entered into the reagent table (.tab file). A dry resin loaded plate is removed from vacuum and installed in the machine for the synthesis.

The modified 96 well polypropylene plate **556** is utilized as the reaction vessel.
15 The working volume in each well is approximately 700 μ l. The bottom of each well is provided with a pressed-fit 20 μ m polypropylene frit and a long capillary exit into a lower collection chamber as is illustrated in Figure 5 of the above referenced United States Patent 5,372,672. The solid support for use in holding the growing oligonucleotide during synthesis is loaded into the wells of the synthesis plate **556** by pipetting the desired
20 volume of a balanced density slurry of the support suspended in an appropriate solvent, typically an acetonitrile-methylene chloride mixture. Reactions can be run on various scales as for instance the above noted 200 nmole and 10 μ mol scales. For oligonucleotide synthesis a CPG support is preferred, however other medium loading polystyrene-PEG supports such as TENTAGEL™ or ARGOGEL™ can also be used.

25 As seen in Figure 24, the synthesis plate is transported back and forth in the X-direction under an array of 8 moveable banks (**530, 532, 534, 536, 538, 540, 542** and **544**) of 8 nozzles (64 total) in the Y-direction, and 6 banks (**544, 546, 548, 550, 552** and **554**) of 48 fixed nozzles, so that each well can receive the appropriate amounts of reagents and/or solvents from any reservoir (large bottle or smaller septa bottle). A sliding balloon-type
30 seal **562** surrounds this nozzle array and joins it to the reaction plate headspace **564**. A slow sweep of nitrogen or argon **522** at ambient pressure across the plate headspace is used

to preserve an anhydrous environment.

The liquid contents in each well do not drip out until the headspace pressure exceeds the capillary forces on the liquid in the exit nozzle. A slight positive pressure in the lower collection chamber can be added to eliminate residual slow leakage from filled wells, or to effect agitation by bubbling inert gas through the suspension. In order to empty the wells, the headspace gas outlet valve is closed and the internal pressure raised to about 2 psi. Normally, liquid contents are blown directly to waste **566**. However, a 96 well microtiter plate can be inserted into the lower chamber beneath the synthesis plate in order to collect the individual well eluents for spectrophotometric monitoring (trityl, etc.) of reaction progress and yield.

The basic plumbing scheme for the machine is the gas-pressurized delivery of reagents. Each reagent is delivered to the synthesis plate through a dedicated supply line, collectively identified at **568**, solenoid valve collectively identified at **570** and nozzle, collectively identified at **572**. Reagents never cross paths until they reach the reaction well. Thus, no line needs to be washed or flushed prior to its next use and there is no possibility of cross-contamination of reagents. The liquid delivery velocity is sufficiently energetic to thoroughly mix the contents within a well to form a homogeneous solution, even when employing solutions having drastically different densities. With this mixing, once reactants are in homogeneous solution, diffusion carries the individual components into and out of the solid support matrix where the desired reaction takes place. Each reagent reservoir can be plumbed to either a single nozzle or any combination of up to 8 nozzles. Each nozzle is also provided with a concentric nozzle washer to wash the outside of the delivery nozzles in order to eliminate problems of crystallized reactant buildup due to slow evaporation of solvent at the tips of the nozzles. The nozzles and supply lines can be primed into a set of dummy wells directly to waste at any time.

The entire plumbing system is fabricated with teflon tubing, and reagent reservoirs are accessed *via* syringe needle/septa or direct connection into the higher capacity bottles. The septum vials **560** are held in removable 8-bottle racks to facilitate easy setup and cleaning. The priming volume for each line is about 350 μ l. The minimum delivery volume is about 2 μ l, and flow rate accuracy is $\pm 5\%$. The actual amount of material delivered depends on a timed flow of liquid. The flow rate for a particular solvent will

depend on its viscosity and wetting characteristics of the teflon tubing. The flow rate (typically 200-350 μ l per sec) is experimentally determined, and this information is contained in the reagent table setup file.

Heating and cooling of the reaction block **526** is effected utilizing a recirculating heat exchanger plate **524**, similar to that found in PCR thermocyclers, that nests with the polypropylene synthesis plate **556** to provide good thermal contact. The liquid contents in a well can be heated or cooled at about 10°C per minute over a range of +5 to +80°C, as polypropylene begins to soften and deform at about 80°C. For temperatures greater than this, a non-disposable synthesis plate machined from stainless steel or monel with replaceable frits can be utilized.

The hardware controller can be any of a wide variety, but conveniently can be designed around a set of three 1 MHz 86332 chips. This controller is used to drive the single X-axis and 8 Y-axis stepper motors as well as provide the timing functions for a total of 154 solenoid valves. Each chip has 16 bidirectional timer I/O and 8 interrupt channels in its timer processing unit (TPU). These are used to provide the step and direction signals, and to read 3 encoder inputs and 2 limit switches for controlling up to three motors per chip. Each 86332 chip also drives a serial chain of 8 UNC5891A darlington array chips to provide power to 64 valves with msec resolution. The controller communicates with the Windows software interface program running on a PC via a 19200 Hz serial channel, and uses an elementary instruction set to communicate valve_number, time_open, motor_number and position_data.

The three components of the software program that run the array synthesizer are the generalized procedure or command (.cmd) file which specifies the synthesis instructions to be performed, the sequence (.seq) file which specifies the scale of the reaction and the order in which variable groups will be added to the core synthon, and the reagent table (.tab) file which specifies the name of a chemical, its location (bottle number), flow rate, and concentration are utilized in conjunction with a basic set of command instructions.

One basic set of command instructions can be:

ADD
IF {block of instructions} END_IF

```

REPEAT      {block of instructions}      END_REPEAT
PRIME, NOZZLE_WASH
WAIT, DRAIN
LOAD, REMOVE
5  NEXT_SEQUENCE
    LOOP_BEGIN, LOOP_END

```

The ADD instruction has two forms, and is intended to have the look and feel of a standard chemical equation. Reagents are specified to be added by a molar amount if the number proceeds the name identifier, or by an absolute volume in microliters if the number follows the identifier. The number of reagents to be added is a parsed list, separated by the “+” sign. For variable reagent identifiers, the key word, <seq>, means look in the sequence table for the identity of the reagent to be added, while the key word, <act>, means add the reagent which is associated with that particular <seq>. Reagents are delivered in the order specified in the list.

15 Thus:

ADD ACN 300

means: Add 300 μ l of the named reagent acetonitrile; ACN to each well of active synthesis

ADD <seq> 300

20 means: If the sequence pointer in the .seq file is to a reagent in the list of reagents, independent of scale, add 300 μ l of that particular reagent specified for that well.

ADD 1.1 PYR + 1.0 <seq> + 1.1 <act1>

25 means: If the sequence pointer in the .seq file is to a reagent in the list of acids in the Class ACIDS_1, and PYR is the name of pyridine, and ethyl chloroformate is defined in the .tab file to activate the class, ACIDS_1, then this instruction means:

Add 1.1 equiv. pyridine

1.0 equiv. of the acid specified for that well and

30 1.1 equiv. of the activator, ethyl chloroformate

The IF command allows one to test what type of reagent is specified in the <seq> variable

and process the succeeding block of commands accordingly.

Thus:

ACYLATION {the procedure name}

BEGIN

```
5          IF CLASS = ACIDS_1
            ADD 1.0 <seq> + 1.1 <act1> + 1.1 PYR
            WAIT 60
            ENDIF
            IF CLASS = ACIDS_2
10          ADD 1.0 <seq> + 1.2 <act1> + 1.2 TEA
            ENDIF
            WAIT 60
            DRAIN 10
            END
```

15 means: Operate on those wells for which reagents contained in the Acid_1 class are specified, WAIT 60 sec, then operate on those wells for which reagents contained in the Acid_2 class are specified, then WAIT 60 sec longer, then DRAIN the whole plate. Note that the Acid_1 group has reacted for a total of 120 sec, while the Acid_2 group has reacted for only 60 sec.

20 The REPEAT command is a simple way to execute the same block of commands multiple times.

Thus:

WASH_1 {the procedure name}

BEGIN

```
25          REPEAT 3
            ADD ACN 300
            DRAIN 15
            END_REPEAT
            END
```

30 means: repeats the add acetonitrile and drain sequence for each well three times.

The PRIME command will operate either on specific named reagents or on nozzles

which will be used in the next associated <seq> operation. The μ l amount dispensed into a prime port is a constant that can be specified in a config.dat file.

5 The NOZZLE_WASH command for washing the outside of reaction nozzles free from residue due to evaporation of reagent solvent will operate either on specific named reagents or on nozzles which have been used in the preceding associated <seq> operation. The machine is plumbed such that if any nozzle in a block has been used, all the nozzles in that block will be washed into the prime port.

The WAIT and DRAIN commands are by seconds, with the drain command applying a gas pressure over the top surface of the plate in order to drain the wells.

10 The LOAD and REMOVE commands are instructions for the machine to pause for operator action.

The NEXT_SEQUENCE command increments the sequence pointer to the next group of substituents to be added in the sequence file. The general form of a .seq file entry is the definition:

15

Well_No	Well_ID	Scale	Sequence
---------	---------	-------	----------

20 The sequence information is conveyed by a series of columns, each of which represents a variable reagent to be added at a particular position. The scale (μ mole) variable is included so that reactions of different scale can be run at the same time if desired. The reagents are defined in a lookup table (the .tab file), which specifies the name of the reagent as referred to in the sequence and command files, its location (bottle number), flow rate, and concentration. This information is then used by the controller software and hardware to determine both the appropriate slider motion to position the plate and slider arms for delivery of a specific reagent, as well as the specific valve and time required to deliver the appropriate reagents. The adept classification of reagents allows the use of conditional IF loops from within a command file to perform addition of different reagents differently during a "single step" performed across 96 wells simultaneously. The special class ACTIVATORS defines certain reagents that always get added with a particular class of reagents (for example tetrazole during a phosphorylation reaction in adding the next nucleotide to a growing oligonucleotide).

25

30

The general form of the .tab file is the definition:

Class	Bottle	Reagent Name	Flow_rate	Conc.
-------	--------	--------------	-----------	-------

5 The LOOP_BEGIN and LOOP_END commands define the block of commands which will continue to operate until a NEXT_SEQUENCE command points past the end of the longest list of reactants in any well.

Not included in the command set is a MOVE command. For all of the above commands, if any plate or nozzle movement is required, this is automatically executed in
10 order to perform the desired solvent or reagent delivery operation. This is accomplished by the controller software and hardware, which determines the correct nozzle(s) and well(s) required for a particular reagent addition, then synchronizes the position of the requisite nozzle and well prior to adding the reagent.

A MANUAL mode can also be utilized in which the synthesis plate and nozzle
15 blocks can be "homed" or moved to any position by the operator, the nozzles primed or washed, the various reagent bottles depressurized or washed with solvent, the chamber pressurized, etc. The automatic COMMAND mode can be interrupted at any point, MANUAL commands executed, and then operation resumed at the appropriate location. The sequence pointer can be incremented to restart a synthesis anywhere within a
20 command file.

In reference to Figure 14, the list of oligonucleotides for synthesis can be rearranged or grouped for optimization of synthesis. Thus at process step 574, the oligonucleotides are grouped according to a factor on which to base the optimization of synthesis. As illustrated in the Examples below, one such factor is the 3' most nucleoside
25 of the oligonucleotide. Using the amidite approach for oligonucleotide synthesis, a nucleotide bearing a 3' phosphoramite is added to the 5' hydroxyl group of a growing nucleotide chain. The first nucleotide (at the 3' terminus of the oligonucleotide - the 3' most nucleoside) is first connected to a solid support. This is normally done batchwise on a large scale as is standard practice during oligonucleotide synthesis.

30 Such solid supports pre-loaded with a nucleoside are commercially available. In utilizing the multi well format for oligonucleotide synthesis, for each oligonucleotide to be

synthesized, an aliquot of a solid support bearing the proper nucleoside thereon is added to the well for synthesis. Prior to loading the sequence of oligonucleotides to be synthesized in the .seq file, they are sorted by the 3' terminal nucleotide. Based on that sorting, all of the oligonucleotide sequences having an "A" nucleoside at their 3' end are grouped
5 together, those with a "C" nucleoside are grouped together as are those with "G" or "T" nucleosides. Thus in loading the nucleoside-bearing solid support into the synthesis wells, machine movements are conserved.

The oligonucleotides can be grouped by the above described parameter or other parameters that facilitate the synthesis of the oligonucleotides. Thus in Figure 14, sorting
10 is noted as being effected by some parameter of type 1, as for instance the above described 3' most nucleoside, or other types of parameters from type 2 to type n at process steps 576, 578 and 580. Since synthesis will be from the 3' end of the oligonucleotides to the 5' end, the oligonucleotide sequences are reverse sorted to read 3' to 5'. The oligonucleotides are entered in the .seq file in this form, i.e., reading 3' to 5'.

15 Once sorted into types, the position of the oligonucleotides on the synthesis plates is specified at process step 582 by the creation of a .seq file as described above. The .seq file is associated with the respective .cmd and .tab files needed for synthesis of the particular chemistries specified for the oligonucleotides at process step 584 by retrieval of the .cmd and .tab files at process step 586 from the sample database 520. These files are
20 then input into the multi well synthesizer at process step 588 for oligonucleotide synthesis. Once physically synthesized, the list of oligonucleotides again enters the general procedure flow as indicated in Figure 1. For shipping, storage or other handling purposes, the plates can be lyophilized at this point if desired. Upon lyophilization, each well contains the oligonucleotides located therein as a dry compound.

25 14. Quality Control.

In an optional step, quality control is performed on the oligonucleotides at process step 600 after a decision is made (decision step 550) to perform quality control. Although optional, quality control may be desired when there is some reason to think that some aspect of the synthetic process step 500 has been compromised. Alternatively, samples of
30 the oligonucleotides may be taken and stored in the event that the results of assays conducted using the oligonucleotides (process step 700) yield confusing results or

suboptimal data. In the latter event, for example, quality control might be performed after decision step **800** if no oligonucleotides with sufficient activity are identified. In either event, decision step **650** follows quality control step process **600**. If one or more of the oligonucleotides do not pass quality control, process step **500** can be repeated, i.e., the
5 oligonucleotides are synthesized for a second time.

The operation of the quality control system general procedure **600** is detailed in steps **610-660** of Figure 15. Also referenced in the following discussion are the robotics and associated analytical instrumentation as shown in Figure 18.

During step **610** (Figure 15), sterile, double-distilled water is transferred by an
10 automated liquid handler (**2040** of Figure 18) to each well of a multi-well plate containing a set of lyophilized antisense oligonucleotides. The automated liquid handler (**2040** of Figure 18) reads the barcode sticker on the multi-well plate to obtain the plate's identification number. Automated liquid handler **2040** then queries Sample Database **520** (which resides in Database Server **2002** of Figure 18) for the quality control assay
15 instruction set for that plate and executes the appropriate steps. Three quality control processes are illustrated, however, it is understood that other quality control processes or steps maybe practiced in addition to or in place of the processes illustrated.

The first illustrative quality control process (steps **622** to **626**) quantitates the concentration of oligonucleotide in each well. If this quality control step is performed, an
20 automated liquid handler (**2040** of Figure 18) is instructed to remove an aliquot from each well of the master plate and generate a replicate daughter plate for transfer to the UV spectrophotometer (**2016** of Figure 18). The UV spectrophotometer (**2016** of Figure 18) then measures the optical density of each well at a wavelength of 260 nanometers. Using standardized conversion factors, a microprocessor within UV spectrophotometer (**2016** of
25 Figure 18) then calculates a concentration value from the measured absorbance value for each well and output the results to Sample Database **520**.

The second illustrative quality control process steps **632** to **636**) quantitates the percent of total oligonucleotide in each well that is full length. If this quality control step is performed, an automated liquid handler (**2040** of Figure 18) is instructed to remove an
30 aliquot from each well of the master plate and generate a replicate daughter plate for transfer to the multichannel capillary gel electrophoresis apparatus (**2022** of Figure 18).

The apparatus electrophoretically resolves in capillary tube gels the oligonucleotide product in each well. As the product reaches the distal end of the tube gel during electrophoresis, a detection window dynamically measures the optical density of the product that passes by it. Following electrophoresis, the value of percent product that passed by the detection window with respect to time is utilized by a built in microprocessor to calculate the relative size distribution of oligonucleotide product in each well. These results are then output to the Sample Database (520).

The third illustrative quality control process steps 632 to 636) quantitates the mass of the oligonucleotide in each well that is full length. If this quality control step is performed, an automated liquid handler (2040 of Figure 18) is instructed to remove an aliquot from each well of the master plate and generate a replicate daughter plate for transfer to the multichannel liquid electrospray mass spectrometer (2018 of Figure 18). The apparatus then uses electrospray technology to inject the oligonucleotide product into the mass spectrometer. A built in microprocessor calculates the mass-to-charge ratio to arrive at the mass of oligonucleotide product in each well. The results are then output to Sample Database 520.

Following completion of the selected quality control processes, the output data is manually examined or is examined using an appropriate algorithm and a decision is made as to whether or not the plate receives "Pass" or "Fail" status. The current criteria for acceptance, for 18 mer oligonucleotides, is that at least 85% of the oligonucleotides in a multi-well plate must be 85% or greater full length product as measured by both capillary gel electrophoresis and mass spectrometry. An input (manual or automated) is then made into Sample Database 520 as to the pass/fail status of the plate. If a plate fails, the process cycles back to step 500, and a new plate of the same oligonucleotides is automatically placed in the plate synthesis request queue (process 554 of Figure 15). If a plate receives "Pass" status, an automated liquid handler (2040 of Figure 18) is instructed to remove appropriate aliquots from each well of the master plate and generate two replicate daughter plates in which the oligonucleotide in each well is at a concentration of 30 micromolar. The plate then moves on to process 700 for oligonucleotide activity evaluation.

15. Cell Lines for Assaying Oligonucleotide Activity. The effect of antisense compounds on target nucleic acid expression can be tested in any of a variety of cell types

provided that the target nucleic acid, or its gene product, is present at measurable levels. This can be routinely determined using, for example, PCR or Northern blot analysis. The following four cell types are provided for illustrative purposes, but other cell types can be routinely used.

5 **T-24 cells:** The transitional cell bladder carcinoma cell line T-24 is obtained from the American Type Culture Collection (ATCC) (Manassas, VA). T-24 cells were routinely cultured in complete McCoy's 5A basal media (Life Technologies, Gaithersburg, MD) supplemented with 10% fetal calf serum, penicillin 100 units per milliliter, and streptomycin 100 micrograms per milliliter (all from Life Technologies).
10 Cells are routinely passaged by trypsinization and dilution when they reach 90% confluence. Cells are routinely seeded into 96-well plates (Falcon-Primaria #3872) at a density of 7000 cells/well for use in RT-PCR analysis. For Northern blotting or other analysis, cells are seeded onto 100 mm or other standard tissue culture plates and treated similarly, using appropriate volumes of medium and oligonucleotide.

15 **A549 cells:** The human lung carcinoma cell line A549 is obtained from the ATCC (Manassas, VA). A549 cells were routinely cultured in DMEM basal media (Life Technologies) supplemented with 10% fetal calf serum, penicillin 100 units per milliliter, and streptomycin 100 micrograms per milliliter (all from Life Technologies). Cells are routinely passaged by trypsinization and dilution when they reach 90% confluence.

20 **NHDF cells:** Human neonatal dermal fibroblast (NHDF) were obtained from the Clonetics Corporation (Walkersville, MD). NHDFs were routinely maintained in Fibroblast Growth Medium (Clonetics Corp.) as provided by the supplier. Cells are maintained for up to 10 passages as recommended by the supplier.

25 **HEK cells:** Human embryonic keratinocytes (HEK) were obtained from the Clonetics Corp. HEKs were routinely maintained in Keratinocyte Growth Medium (Clonetics Corp.) as provided by the supplier. Cell are routinely maintained for up to 10 passages as recommended by the supplier.

16. Treatment of Cells with Candidate Compounds:

30 When cells reach about 80% confluency, they are treated with oligonucleotide. For cells grown in 96-well plates, wells are washed once with 200 μ l OPTI-MEM-1™ reduced-serum medium (Life Technologies) and then treated with 130 μ l of OPTI-MEM-

1TM containing 3.75 μ g/ml LIPOFECTINTM (Life Technologies) and the desired oligonucleotide at a final concentration of 150 nM. After 4 hours of treatment, the medium was replaced with fresh medium. Cells were harvested 16 hours after oligonucleotide treatment.

5 Alternatively, for cells resistant to cationic mediated transfection, oligonucleotides can be introduced by electroporation. Electroporation conditions must be optimized for every cell type. In general, oligonucleotide is added directly to complete growth media to a final concentration between 1 and 20 micromolar. An electronic pulse is delivered to the cells using a BTX T820 ELECTRO SQUARE PORATORTM using a Multi-coaxial 96-
10 well electrode (BT840) (BTX Corporation, San Diego, California). Following electroporation, the cells are returned to the incubator for 16 hours.

17. Assaying Oligonucleotide Activity:

 Oligonucleotide-mediated modulation of expression of a target nucleic acid can be assayed in a variety of ways known in the art. For example, target RNA levels can be
15 quantitated by, e.g., Northern blot analysis, competitive PCR, or reverse transcriptase polymerase chain reaction (RT-PCR). RNA analysis can be performed on total cellular RNA or, preferably in the case of polypeptide-encoding nucleic acids, poly(A)+ mRNA. For RT-PCR, poly(A)+ mRNA is preferred. Methods of RNA isolation are taught in, for example, Ausubel *et al.* (*Short Protocols in Molecular Biology*, 2nd Ed., pp. 4-1 to 4-13,
20 Greene Publishing Associates and John Wiley & Sons, New York, 1992). Northern blot analysis is routine in the art (*Id.*, pp. 4-14 to 4-29).

 Alternatively, total RNA can be prepared from cultured cells or tissue using the QIAGEN RNeasy®-96 kit for the high throughput preparation of RNA (QIAGEN, Inc., Valencia, CA). Essentially, protocols are carried out according to the manufacturer's
25 directions. Optionally, a DNase step is included to remove residual DNA prior to RT-PCR.

 To improve efficiency and accuracy the repetitive pipeting steps and elution step have been automated using a QIAGEN Bio-Robot 9604. Essentially after lysing of the oligonucleotide treated cell cultures in situ, the plate is transferred to the robot deck where
30 the pipeting, DNase treatment, and elution steps are carried out.

 Reverse transcriptase polymerase chain reaction (RT-PCR) can be conveniently

accomplished using the commercially available ABI PRISM® 7700 Sequence Detection System (PE-Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Other methods of PCR are also known in the art.

5 Target protein levels can be quantitated in a variety of ways well known in the art, such as immunoprecipitation, Western blot analysis (immunoblotting), Enzyme-linked immunosorbent assay (ELISA) or fluorescence-activated cell sorting (FACS). Antibodies directed to a protein encoded by a target nucleic acid can be identified and obtained from a variety of sources, such as the MSRS catalog of antibodies, (Aerie Corporation, Birmingham, MI or via the internet at <http://www.ANTIBODIES-PROBES.com/>), or can
10 be prepared via conventional antibody generation methods. Methods for preparation of polyclonal, monospecific ("antipeptide") and monoclonal antisera are taught by, for example, Ausubel *et al.* (*Short Protocols in Molecular Biology*, 2nd Ed., pp. 11-3 to 11-54, Greene Publishing Associates and John Wiley & Sons, New York, 1992).

Immunoprecipitation methods are standard in the art and are described by, for
15 example, Ausubel *et al.* (*Id.*, pp. 10-57 to 10-63). Western blot (immunoblot) analysis is standard in the art (*Id.*, pp. 10-32 to 10-10-35). Enzyme-linked immunosorbent assays (ELISA) are standard in the art (*Id.*, pp. 11-5 to 11-17).

Because it is preferred to assay the compounds of the invention in a batchwise fashion, i.e., in parallel to the automated synthesis process described above, preferred
20 means of assaying are suitable for use in 96-well plates and with robotic means. Accordingly, automated RT-PCR is preferred for assaying target nucleic acid levels, and automated ELISA is preferred for assaying target protein levels.

The assaying step, general procedure step **700**, is described in detail in Figure 16. After an appropriate cell line is selected at process step **710**, a decision is made at decision
25 step **714** as to whether RT-PCR will be the only method by which the activity of the compounds is evaluated. In some instances, it is desirable to run alternative assay methods at process step **718**; for example, when it is desired to assess target polypeptide levels as well as target RNA levels, an immunoassay such as an ELISA is run in parallel with the RT-PCR assays. Preferably, such assays are tractable to semi-automated or robotic means.

30 When RT-PCR is used to evaluate the activities of the compounds, cells are plated into multi-well plates (typically, 96-well plates) in process step **720** and treated with test or

control oligonucleotides in process step 730. Then, the cells are harvested and lysed in process step 740 and the lysates are introduced into an apparatus where RT-PCR is carried out in process step 750. A raw data file is generated, and the data is downloaded and compiled at step 760. Spreadsheet files with data charts are generated at process step 770, and the experimental data is analyzed at process step 780. Based on the results, a decision is made at process step 785 as to whether it is necessary to repeat the assays and, if so, the process begins again with step 720. In any event, data from all the assays on each oligonucleotide are compiled and statistical parameters are automatically determined at process step 790.

18. Classification of Compounds Based on Their Activity:

Following assaying, general procedure step 700, oligonucleotide compounds are classified according to one or more desired properties. Typically, three classes of compounds are used: active compounds, marginally active (or “marginal”) compounds and inactive compounds. To some degree, the selection criteria for these classes vary from target to target, and members of one or more classes may not be present for a given set of oligonucleotides.

However, some criteria are constant. For example, inactive compounds will typically comprise those compounds having 5% or less inhibition of target expression (relative to basal levels). Active compounds will typically cause at least 30% inhibition of target expression, although lower levels of inhibition are acceptable in some instances. Marginal compounds will have activities intermediate between active and inactive compounds, with preferred marginal compounds having activities more like those of active compounds.

19. Optimization of Lead Compounds by Sequence.

One means by which oligonucleotide compounds are optimized for activity is by varying their nucleobase sequences so that different regions of the target nucleic acid are targeted. Some such regions will be more accessible to oligonucleotide compounds than others, and “sliding” a nucleobase sequence along a target nucleic acid only a few bases can have significant effects on activity. Accordingly, varying or adjusting the nucleobase sequences of the compounds of the invention is one means by which suboptimal compounds can be made optimal, or by which new active compounds can be generated.

The operation of the gene walk process 1100 detailed in steps 1104-1112 of Figure 17 is detailed as follows. As used herein, the term "gene walk" is defined as the process by which a specified oligonucleotide sequence x that binds to a specified nucleic acid target y is used as a frame of reference around which a series of new oligonucleotides sequences capable of hybridizing to nucleic acid target y are generated that are sequence shifted increments of oligonucleotide sequence x . Gene walking can be done "downstream", "upstream" or in both directions from a specified oligonucleotide.

During step 1104 the user manually enters the identification number of the oligonucleotide sequence around which it is desired to execute gene walk process 1100 and the name of the corresponding target nucleic acid. The user then enters the scope of the gene walk at step 1104, by which is meant the number of oligonucleotide sequences that it is desired to generate. The user then enters in step 1108 a positive integer value for the sequence shift increment. Once this data is generated, the gene walk is effected. This causes a subroutine to be executed that automatically generates the desired list of sequences by walking along the target sequence. At that point, the user proceeds to process 400 to assign chemistries to the selected oligonucleotides.

Example 16 below, details a gene walk. In subsequent steps, this new set of nucleobase sequences generated by the gene walk is used to direct the automated synthesis at general procedure step 500 of a second set of candidate oligonucleotides. These compounds are then taken through subsequent process steps to yield active compounds or reiterated as necessary to optimize activity of the compounds.

20. Optimization of Lead Compounds by Chemistry.

Another means by which oligonucleotide compounds of the invention are optimized is by reiterating portions of the process of the invention using marginal or active compounds from the first iteration and selecting additional chemistries to the nucleobase sequences thereof.

Thus, for example, an oligonucleotide chemistry different from that of the first set of oligonucleotides is assigned at general procedure step 400. The nucleobase sequences of marginal compounds are used to direct the synthesis at general procedure step 500 of a second set of oligonucleotides having the second assigned chemistry. The resulting second set of oligonucleotide compounds is assayed in the same manner as the first set at

procedure process step **700** and the results are examined to determine if compounds having sufficient activity have been generated at decision step **800**.

21. Identification of Sites Amenable to Antisense Technologies.

In a related process, a second oligonucleotide chemistry is assigned at procedure
5 step **400** to the nucleobase sequences of all of the oligonucleotides (or, at least, all of the active and marginal compounds) and a second set of oligonucleotides is synthesized at procedure step **500** having the same nucleobase sequences as the first set of compounds. The resulting second set of oligonucleotide compounds is assayed in the same manner as the first set at procedure step **700** and active and marginal compounds are identified at
10 procedure steps **800** and **1000**.

In order to identify sites on the target nucleic acid that are amenable to a variety of antisense technologies, the following mathematically simple steps are taken. The sequences of active and marginal compounds from two or more such automated syntheses/assays are compared and a set of nucleobase sequences that are active, or
15 marginally so, in both sets of compounds is identified. The reverse complements of these nucleobase sequences corresponds to sequences of the target nucleic acid that are tractable to a variety of antisense and other sequence-based technologies. These antisense-sensitive sites are assembled into contiguous sequences (contigs) using the procedures described for assembling target nucleotide sequences (at procedure step **200**).

22. Systems for Executing Preferred Methods of the Invention.

An embodiment of computer, network and instrument resources for effecting the methods of the invention is shown in Figure 18. In this embodiment, four computer servers are provided. First, a large database server **2002** stores all chemical structure, sample tracking and genomic, assay, quality control, and program status data. Further, this
25 database server serves as the platform for a document management system. Second, a compute engine **2004** runs computational programs including RNA folding, oligonucleotide walking, and genomic searching. Third, a file server **2006** allows raw instrument output storage and sharing of robot instructions. Fourth, a groupware server **2008** enhances staff communication and process scheduling.

A redundant high-speed network system is provided between the main servers and
30 the bridges **2026**, **2028** and **2030**. These bridges provide reliable network access to the

many workstations and instruments deployed for this process. The instruments selected to support this embodiment are all designed to sample directly from standard 96 well microtiter plates, and include an optical density reader **2016**, a combined liquid chromatography and mass spectroscopy instrument **2018**, a gel fluorescence and scintillation imaging system **2032** and **2042**, a capillary gel electrophoreses system **2022** and a real-time PCR system **2034**.

Most liquid handling is accomplished automatically using robots with individually controllable robotic pipetters **2038** and **2020** as well as a 96-well pipette system **2040** for duplicating plates. Windows NT or Macintosh workstations **2044**, **2024**, and **2036** are deployed for instrument control, analysis and productivity support.

23. Relational Database.

Data is stored in an appropriate database. For use with the methods of the invention, a relational database is preferred. Figure 19 illustrates the data structure of a sample relational database. Various elements of data are segregated among linked storage elements of the database.

EXAMPLES

The following examples illustrate the invention and are not intended to limit the same. Those skilled in the art will recognize, or be able to ascertain through routine experimentation, numerous equivalents to the specific procedures, materials and devices described herein. Such equivalents are considered to be within the scope of the present invention.

EXAMPLE 1: Selection of CD40 as a Target

Cell-cell interactions are a feature of a variety of biological processes. In the activation of the immune response, for example, one of the earliest detectable events in a normal inflammatory response is adhesion of leukocytes to the vascular endothelium, followed by migration of leukocytes out of the vasculature to the site of infection or injury. The adhesion of leukocytes to vascular endothelium is an obligate step in their migration out of the vasculature (for a review, see Albelda *et al.*, *FASEB J.*, 1994, 8, 504). As is well known in the art, cell-cell interactions are also critical for propagation of both B-

lymphocytes and T-lymphocytes resulting in enhanced humoral and cellular immune responses, respectively (for a reviews, see Makgoba *et al.*, *Immunol. Today*, 1989, 10, 417; Janeway, *Sci. Amer.*, 1993, 269, 72).

CD40 was first characterized as a receptor expressed on B-lymphocytes. It was later found that engagement of B-cell CD40 with CD40L expressed on activated T-cells is essential for T-cell dependent B-cell activation (i.e. proliferation, immunoglobulin secretion, and class switching) (for a review, see Gruss *et al. Leuk. Lymphoma*, 1997, 24, 393). A full cDNA sequence for CD40 is available (GenBank accession number X60592, incorporated herein by reference as SEQ ID NO:85).

As interest in CD40 mounted, it was subsequently revealed that functional CD40 is expressed on a variety of cell types other than B-cells, including macrophages, dendritic cells, thymic epithelial cells, Langerhans cells, and endothelial cells (*Ibid.*). These studies have led to the current belief that CD40 plays a much broader role in immune regulation by mediating interactions of T-cells with cell types other than B-cells. In support of this notion, it has been shown that stimulation of CD40 in macrophages and dendritic results is required for T-cell activation during antigen presentation (*Id.*). Recent evidence points to a role for CD40 in tissue inflammation as well. Production of the inflammatory mediators IL-12 and nitric oxide by macrophages has been shown to be CD40 dependent (Buhlmann *et al.*, *J. Clin. Immunol.*, 1996, 16, 83). In endothelial cells, stimulation of CD40 by CD40L has been found to induce surface expression of E-selectin, ICAM-1, and VCAM-1, promoting adhesion of leukocytes to sites of inflammation (Buhlmann *et al.*, *J. Clin. Immunol*, 1996, 16, 83; Gruss *et al.*, *Leuk Lymphoma*, 1997, 24, 393). Finally, a number of reports have documented overexpression of CD40 in epithelial and hematopoietic tumors as well as tumor infiltrating endothelial cells, indicating that CD40 may play a role in tumor growth and/or angiogenesis as well (Gruss *et al.*, *Leuk Lymphoma*, 1997, 24, 393-422; Kluth *et al. Cancer Res*, 1997, 57, 891).

Due to the pivotal role that CD40 plays in humoral immunity, the potential exists that therapeutic strategies aimed at downregulating CD40 may provide a novel class of agents useful in treating a number of immune associated disorders, including but not limited to graft versus host disease, graft rejection, and autoimmune diseases such as multiple sclerosis, systemic lupus erythematosus, and certain forms of arthritis. Inhibitors

of CD40 may also prove useful as an anti-inflammatory compound, and could therefore be useful as treatment for a variety of diseases with an inflammatory component such as asthma, rheumatoid arthritis, allograft rejections, inflammatory bowel disease, and various dermatological conditions, including psoriasis. Finally, as more is learned about the association between CD40 overexpression and tumor growth, inhibitors of CD40 may prove useful as anti-tumor agents as well.

Currently, there are no known therapeutic agents which effectively inhibit the synthesis of CD40. To date, strategies aimed at inhibiting CD40 function have involved the use of a variety of agents that disrupt CD40/CD40L binding. These include monoclonal antibodies directed against either CD40 or CD40L, soluble forms of CD40, and synthetic peptides derived from a second CD40 binding protein, A20. The use of neutralizing antibodies against CD40 and/or CD40L in animal models has provided evidence that inhibition of CD40 stimulation would have therapeutic benefit for GVHD, allograft rejection, rheumatoid arthritis, SLE, MS, and B-cell lymphoma (Buhlmann *et al.*, *J. Clin. Immunol.*, 1996, 16, 83). However, due to the expense, short half-life, and bioavailability problems associated with the use of large proteins as therapeutic agents, there is a long felt need for additional agents capable of effectively inhibiting CD40 function. Oligonucleotides compounds avoid many of the pitfalls of current agents used to block CD40/CD40L interactions and may therefore prove to be uniquely useful in a number of therapeutic applications.

EXAMPLE 2: Generation of Virtual Oligonucleotides Targeted to CD40

The process of the invention was used to select oligonucleotides targeted to CD40, generating the list of oligonucleotide sequences with desired properties as shown in Figure 22. From the assembled CD40 sequence, the process began with determining the desired oligonucleotide length to be eighteen nucleotides, as represented in step 2500. All possible oligonucleotides of this length were generated by Oligo 5.0™, as represented in step 2504. Desired thermodynamic properties were selected in step 2508. The single parameter used was oligonucleotides of melting temperature less than or equal to 40°C were discarded. In step 2512, oligonucleotide melting temperatures were calculated by Oligo 5.0™. Oligonucleotide sequences possessing an undesirable score were discarded. It

is believed that oligonucleotides with melting temperatures near or below physiological and cell culture temperatures will bind poorly to target sequences. All oligonucleotide sequences remaining were exported into a spreadsheet. In step 2516, desired sequence properties are selected. These include discarding oligonucleotides with at least one stretch of four guanosines in a row and stretches of six of any other nucleotide in a row. In step 2520, a spreadsheet macro removed all oligonucleotides containing the text string "GGGG." In step 2524, another spreadsheet macro removed all oligonucleotides containing the text strings "AAAAAA" or "CCCCCC" or "TTTTTT." From the remaining oligonucleotide sequences, 84 sequences were selected manually with the criteria of having an uniform distribution of oligonucleotide sequences throughout the target sequence, as represented in step 2528. These oligonucleotide sequences were then passed to the next step in the process, assigning actual oligonucleotide chemistries to the sequences.

EXAMPLE 3: Input Files For Automated Oligonucleotide Synthesis Command File (.cmd File)

Table 2 is a command file for synthesis of an oligonucleotide having regions of 2'-O-(2-methoxyethyl) nucleosides and a central region of 2'-deoxy nucleosides each linked by phosphorothioate internucleotide linkages.

Table 2

```
SOLID_SUPPORT_SKIP
      BEGIN
                Next_Sequence
      END

INITIAL-WASH
      BEGIN
                Add ACN 300
                Drain 10
      END
```


LOOP-BEGIN

DEBLOCK

BEGIN

Prime TCA

5

Load Tray

Repeat 2

Add TCA 150

Wait 10

Drain 8

10

End_Repeat

Remove Tray

Add TCA 125

Wait 10

Drain 8

15

END

WASH_AFTER_DEBLOCK

BEGIN

Repeat 3

Add ACN 250 To_All

20

Drain 10

End_Repeat

END

COUPLING

BEGIN

25

if class = DEOXY_THIOATE

Nozzle wash <act1>

prime <act1>

prime <seq>

Add <act1> 70 + <seq> 70

```

    Wait 40
        Drain 5
    end-if
    if class = MOE_THIOATE
5      Nozzle wash <act1>
        Prime <act1>
        prime <seq>
        Add <act1> 120 + <seq> 120
        Wait 230
10     Drain 5
    End_if
END
```

WASH_AFTER_COUPLING

```

    BEGIN
15      Add ACN 200 To_All
        Drain 10
    END
```

OXIDIZE

```

20      BEGIN
        if class = DEOXY_THIOATE
        Add BEAU 180
        Wait 40
        Drain 7
25      end_if
        if class = MOE_THIOATE
        Add BEAU 200
        Wait 120
        Drain 7
30      end_if
```

END

CAP

BEGIN

5 Add CAP_B 80 + CAP_A 80
 Wait 20
 Drain 7

END

10 WASH_AFTER_CAP

BEGIN

 Add ACN 150 To_All
 Drain 5
 Add ACN 250 To_All
15 Drain 11

END

BASE_COUNTER

BEGIN

20 Next_Sequence
 END

LOOP_END

DEBLOCK_FINAL

25 BEGIN

 Prime TCA
 Load Tray
 Repeat 2
 Add TCA 150 To_All
30 Wait 10

Drain 8

End_Repeat

Remove Tray

Add TCA 125 To_All

5

Wait 10

Drain 10

END

FINAL_WASH

BEGIN

10

Repeat 4

Add ACN 300 to_All

Drain_12

End_Repeat

END

15

ENDALL

BEGIN

Wait 3

END

Sequence files (.seq Files)

20

Table 3 is a .seq file for oligonucleotides having 2'-deoxy nucleosides linked by phosphorothioate internucleotide linkages.

Table 3

Identity of columns: **Syn #, Well, Scale, Nucleotide at particular position** (identified using base identifier followed by backbone identifier where "s" is phosphorothioate).

25

Note the columns wrap around to next line when longer than one line.

1	A01	200	As	Cs	Cs	As	Gs	Gs	As	Cs	Gs
Gs	Cs	Gs	Gs	As	Cs	Cs	As	G			

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59

PATENT

5	2	A02	200	As	Cs	Gs	Gs	Cs	Gs	Gs	As	Cs
	Cs	As	Gs	As	Gs	Ts	Gs	Gs	A			
	3	A03	200	As	Cs	Cs	As	As	Gs	Cs	As	Gs
	As	Cs	Gs	Gs	As	Gs	As	Cs	G			
	4	A04	200	As	Gs	Gs	As	Gs	As	Cs	Cs	Cs
10	Cs	Gs	As	Cs	Gs	As	As	Cs	G			
	5	A05	200	As	Cs	Cs	Cs	Cs	Gs	As	Cs	Gs
	As	As	Cs	Gs	As	Cs	Ts	Gs	G			
	6	A06	200	As	Cs	Gs	As	As	Cs	Gs	As	Cs
	Ts	Gs	Gs	Cs	Gs	As	Cs	As	G			
15	7	A07	200	As	Cs	Gs	As	Cs	Ts	Gs	Gs	Cs
	Gs	As	Cs	As	Gs	Gs	Ts	As	G			
	8	A08	200	As	Cs	As	Gs	Gs	Ts	As	Gs	Gs
	Ts	Cs	Ts	Ts	Gs	Gs	Ts	Gs	G			
	9	A09	200	As	Gs	Gs	Ts	Cs	Ts	Ts	Gs	Gs
20	Ts	Gs	Gs	Gs	Ts	Gs	As	Cs	G			
	10	A10	200	As	Gs	Ts	Cs	As	Cs	Gs	As	Cs
	As	As	Gs	As	As	As	Cs	As	C			
	11	A11	200	As	Cs	Gs	As	Cs	As	As	Gs	As
	As	As	Cs	As	Cs	Gs	Gs	Ts	C			
25	12	A12	200	As	Gs	As	As	As	Cs	As	Cs	Gs
	Gs	Ts	Cs	Gs	Gs	Ts	Cs	Cs	T			
	13	B01	200	As	As	Cs	As	Cs	Gs	Gs	Ts	Cs
	Gs	Gs	Ts	Cs	Cs	Ts	Gs	Ts	C			
	14	B02	200	As	Cs	Ts	Cs	As	Cs	Ts	Gs	As
30	Cs	Gs	Ts	Gs	Ts	Cs	Ts	Cs	A			
	15	B03	200	As	Cs	Gs	Gs	As	As	Gs	Gs	As
	As	Cs	Gs	Cs	Cs	As	Cs	Ts	T			
	16	B04	200	As	Ts	Cs	Ts	Gs	Ts	Gs	Gs	As
	Cs	Cs	Ts	Ts	Gs	Ts	Cs	Ts	C			
	17	B05	200	As	Cs	As	Cs	Ts	Ts	Cs	Ts	Ts

ISIS-3455 is a patent application for a method of determining the relative positions of a set of points in a 3D space. The method involves determining the relative positions of a set of points in a 2D space, and then determining the relative positions of the points in a 3D space. The method is described in detail in the patent application.

5	Cs	Cs	Gs	As	Cs	Cs	Gs	Ts	G			
	18	B06	200	As	Cs	Ts	Cs	Ts	Cs	Gs	As	Cs
	As	Cs	As	Gs	Gs	As	Cs	Gs	T			
	19	B07	200	As	As	As	Cs	Cs	Cs	Cs	As	Gs
	Ts	Ts	Cs	Gs	Ts	Cs	Ts	As	A			
10	20	B08	200	As	Ts	Gs	Ts	Cs	Cs	Cs	Cs	As
	As	As	Gs	As	Cs	Ts	As	Ts	G			
	21	B09	200	As	Cs	Gs	Cs	Ts	Cs	Gs	Gs	Gs
	As	Cs	Gs	Gs	Gs	Ts	Cs	As	G			
	22	B10	200	As	Gs	Cs	Cs	Gs	As	As	Gs	As
15	As	Gs	As	Gs	Gs	Ts	Ts	As	C			
	23	B11	200	As	Cs	As	Cs	As	Gs	Ts	As	Gs
	As	Cs	Gs	As	As	As	Gs	Cs	T			
	24	B12	200	As	Cs	As	Cs	Ts	Cs	Ts	Gs	Gs
	Ts	Ts	Ts	Cs	Ts	Gs	Gs	As	C			
20	25	C01	200	As	Cs	Gs	As	Cs	Cs	As	Gs	As
	As	As	Ts	As	Gs	Ts	Ts	Ts	T			
	26	C02	200	As	Gs	Ts	Ts	As	As	As	As	Gs
	Gs	Gs	Cs	Ts	Gs	Cs	Ts	As	G			
	27	C03	200	As	Gs	Gs	Ts	Ts	Gs	Ts	Gs	As
25	Cs	Gs	As	Cs	Gs	As	Gs	Gs	T			
	28	C04	200	As	As	Ts	Gs	Ts	As	Cs	Cs	Ts
	As	Cs	Gs	Gs	Ts	Ts	Gs	Gs	C			
	29	C05	200	As	Gs	Ts	Cs	As	Cs	Gs	Ts	Cs
	Cs	Ts	Cs	Ts	Cs	Ts	Gs	Ts	C			
30	30	C06	200	Cs	Ts	Gs	Gs	Cs	Gs	As	Cs	As
	Gs	Gs	Ts	As	Gs	Gs	Ts	Cs	T			
	31	C07	200	Cs	Ts	Cs	Ts	Gs	Ts	Gs	Ts	Gs
	As	Cs	Gs	Gs	Ts	Gs	Gs	Ts	C			
	32	C08	200	Cs	As	Gs	Gs	Ts	Cs	Gs	Ts	Cs
	Ts	Ts	Cs	Cs	Cs	Gs	Ts	Gs	G			

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5	33	C09	200	Cs	Ts	Gs	Ts	Gs	Gs	Ts	As	Gs
	As	Cs	Gs	Ts	Gs	Gs	As	Cs	A			
	34	C10	200	Cs	Ts	As	As	Cs	Gs	As	Ts	Gs
	Ts	Cs	Cs	Cs	Cs	As	As	As	G			
	35	C11	200	Cs	Ts	Gs	Ts	Ts	Cs	Gs	As	Cs
10	As	Cs	Ts	Cs	Ts	Gs	Gs	Ts	T			
	36	C12	200	Cs	Ts	Gs	Gs	As	Cs	Cs	As	As
	Cs	As	Cs	Gs	Ts	Ts	Gs	Ts	C			
	37	D01	200	Cs	Cs	Gs	Ts	Cs	Cs	Gs	Ts	Gs
	Ts	Ts	Ts	Gs	Ts	Ts	Cs	Ts	G			
15	38	D02	200	Cs	Ts	Gs	As	Cs	Ts	As	Cs	As
	As	Cs	As	Gs	As	Cs	As	Cs	C			
	39	D03	200	Cs	As	As	Cs	As	Gs	As	Cs	As
	Cs	Cs	As	Gs	Gs	Gs	Gs	Ts	C			
	40	D04	200	Cs	As	Gs	Gs	Gs	Gs	Ts	Cs	Cs
20	Ts	As	Gs	Cs	Cs	Gs	As	Cs	T			
	41	D05	200	Cs	Ts	Cs	Ts	As	Gs	Ts	Ts	As
	As	As	As	Gs	Gs	Gs	Cs	Ts	G			
	42	D06	200	Cs	Ts	Gs	Cs	Ts	As	Gs	As	As
	Gs	Gs	As	Cs	Cs	Gs	As	Gs	G			
25	43	D07	200	Cs	Ts	Gs	As	As	As	Ts	Gs	Ts
	As	Cs	Cs	Ts	As	Cs	Gs	Gs	T			
	44	D08	200	Cs	As	Cs	Cs	Cs	Gs	Ts	Ts	Ts
	Gs	Ts	Cs	Cs	Gs	Ts	Cs	As	A			
	45	D09	200	Cs	Ts	Cs	Gs	As	Ts	As	Cs	Gs
30	Gs	Gs	Ts	Cs	As	Gs	Ts	Cs	A			
	46	D10	200	Gs	Gs	Ts	As	Gs	Gs	Ts	Cs	Ts
	Ts	Gs	Gs	Ts	Gs	Gs	Gs	Ts	G			
	47	D11	200	Gs	As	Cs	Ts	Ts	Ts	Gs	Cs	Cs
	Ts	Ts	As	Cs	Gs	Gs	As	As	G			
	48	D12	200	Gs	Ts	Gs	Gs	As	Gs	Ts	Cs	Ts

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			Ts	Ts	Gs	Ts	Cs	Ts	Gs	Ts	G			
	49	E01	200	Gs	Gs	As	Gs	Ts	Cs	Ts	Ts	Ts		
				Gs	Ts	Cs	Ts	Gs	Ts	Gs	T			
	50	E02	200	Gs	Gs	As	Cs	As	Cs	Ts	Cs	Ts		
5				Cs	Gs	As	Cs	As	Gs	G				
	51	E03	200	Gs	As	Cs	As	Cs	As	Gs	Gs	As		
				Cs	Gs	Ts	Gs	Gs	Cs	Gs	As	G		
	52	E04	200	Gs	As	Gs	Ts	As	Cs	Gs	As	Gs		
				Cs	Gs	Gs	Gs	Cs	Cs	Gs	As	A		
10				53	E05	200	Gs	As	Cs	Ts	As	Ts	Gs	Gs
				As	Gs	As	Cs	Gs	Cs	Ts	Cs	G		
	54	E06	200	Gs	As	As	Gs	As	Gs	Gs	Gs	Ts	Ts	
				As	Cs	As	Cs	As	Gs	Ts	As	G		
	55	E07	200	Gs	As	Gs	Gs	Ts	Ts	As	Cs	As		
15				Cs	As	Gs	Ts	As	Gs	As	Cs	G		
	56	E08	200	Gs	Ts	Ts	Gs	Ts	Cs	Cs	Gs	Ts		
				Cs	Cs	Gs	Ts	Gs	Ts	Ts	Ts	G		
	57	E09	200	Gs	As	Cs	Ts	Cs	Ts	Cs	Gs	Gs		
				Gs	As	Cs	Cs	As	Cs	As	C			
20				58	E10	200	Gs	Ts	As	Gs	Gs	As	Gs	As
				Cs	Cs	As	Cs	Gs	As	Cs	Cs	A		
	59	E11	200	Gs	Gs	Ts	Ts	Cs	Ts	Ts	Cs	Gs		
				Gs	Ts	Ts	Gs	Gs	Ts	Ts	As	T		
	60	E12	200	Gs	Ts	Gs	Gs	Gs	Gs	Ts	Ts	Cs		
25				Gs	Ts	Cs	Cs	Ts	Ts	Gs	Gs	G		
	61	F01	200	Gs	Ts	Cs	As	Cs	Gs	Ts	Cs	Cs		
				Ts	Cs	Ts	Gs	As	As	As	Ts	G		
	62	F02	200	Gs	Ts	Cs	Cs	Ts	Cs	Cs	Ts	As		
				Cs	Cs	Gs	Ts	Ts	Ts	Cs	Ts	C		
30				63	F03	200	Gs	Ts	Cs	Cs	Cs	As	Cs	Gs
				Ts	Cs	Cs	Gs	Ts	Cs	Ts	Ts	C		

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5	64	F04	200	Ts	Cs	As	Cs	Cs	As	Gs	Gs	As
	Cs	Gs	Gs	Cs	Gs	Gs	As	Cs	C			
	65	F05	200	Ts	As	Cs	Cs	As	As	Gs	Cs	As
	Gs	As	Cs	Gs	Gs	As	Gs	As	C			
	66	F06	200	Ts	Cs	Cs	Ts	Gs	Ts	Cs	Ts	Ts
10	Ts	Gs	As	Cs	Cs	As	Cs	Ts	C			
	67	F07	200	Ts	Gs	Ts	Cs	Ts	Ts	Ts	Gs	As
	Cs	Cs	As	Cs	Ts	Cs	As	Cs	T			
	68	F08	200	Ts	Gs	As	Cs	Cs	As	Cs	Ts	Cs
	As	Cs	Ts	Gs	As	Cs	Gs	Ts	G			
15	69	F09	200	Ts	Gs	As	Cs	Gs	Ts	Gs	Ts	Cs
	Ts	Cs	As	As	Gs	Ts	Gs	As	C			
	70	F10	200	Ts	Cs	As	As	Gs	Ts	Gs	As	Cs
	Ts	Ts	Ts	Gs	Cs	Cs	Ts	Ts	A			
	71	F11	200	Ts	Gs	Ts	Ts	Ts	As	Ts	Gs	As
20	Cs	Gs	Cs	Ts	Gs	Gs	Gs	Gs	T			
	72	F12	200	Ts	Ts	As	Ts	Gs	As	Cs	Gs	Cs
	Ts	Gs	Gs	Gs	Gs	Ts	Ts	Gs	G			
	73	G01	200	Ts	Gs	As	Cs	Gs	Cs	Ts	Gs	Gs
	Gs	Gs	Ts	Ts	Gs	Gs	As	Ts	C			
25	74	G02	200	Ts	Cs	Gs	Ts	Cs	Ts	Ts	Cs	Cs
	Cs	Gs	Ts	Gs	Gs	As	Gs	Ts	C			
	75	G03	200	Ts	Gs	Gs	Ts	As	Gs	As	Cs	Gs
	Ts	Gs	Gs	As	Cs	As	Cs	Ts	T			
	76	G04	200	Ts	Ts	Cs	Ts	Ts	Cs	Cs	Gs	As
30	Cs	Cs	Gs	Ts	Gs	As	Cs	As	T			
	77	G05	200	Ts	Gs	Gs	Ts	As	Gs	As	Cs	Gs
	Cs	Ts	Cs	Gs	Gs	Gs	As	Cs	G			
	78	G06	200	Ts	As	Gs	As	Cs	Gs	Cs	Ts	Cs
	Gs	Gs	Gs	As	Cs	Gs	Gs	Gs	T			
	79	G07	200	Ts	Ts	Ts	Ts	As	Cs	As	Gs	Ts

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	Gs	Gs	Gs	As	As	Cs	Cs	Ts	G			
	80	G08	200	Ts	Gs	Gs	Gs	As	As	Cs	Cs	Ts
	Gs	Ts	Ts	Cs	Gs	As	Cs	As	C			
	81	G09	200	Ts	Cs	Gs	Gs	Gs	As	Cs	Cs	As
5	Cs	Cs	As	Cs	Ts	As	Gs	Gs	G			
	82	G10	200	Ts	As	Gs	Gs	As	Cs	As	As	As
	Cs	Gs	Gs	Ts	As	Gs	Gs	As	G			
	83	G11	200	Ts	Gs	Cs	Ts	As	Gs	As	As	Gs
	Gs	As	Cs	Cs	Gs	As	Gs	Gs	T			
10	84	G12	200	Ts	Cs	Ts	Gs	Ts	Cs	As	Cs	Ts
	Cs	Cs	Gs	As	Cs	Gs	Ts	Gs	G			

Table 4 is a .seq file for oligonucleotides having regions of 2'-O-(2-methoxyethyl)-nucleosides and a central region of 2'-deoxy nucleosides each linked by phosphorothioate internucleotide linkages.

15

Table 4

Identity of columns: **Syn #, Well, Scale, Nucleotide at particular position** (identified using base identifier followed by backbone identifier where "s" is phosphorothioate and "moe" indicated a 2'-O-(2-methoxyethyl) substituted nucleoside). The columns wrap around to next line when longer than one line.

20

```

1 A01 200 moeAs moeCs moeCs moeAs Gs Gs As Cs Gs Gs Cs Gs Gs As
moeCs moeCs moeAs moeG
2 A02 200 moeAs moeCs moeGs moeGs Cs Gs Gs As Cs Cs As Gs As Gs
moeTs moeGs moeGs moeA
25 3 A03 200 moeAs moeCs moeCs moeAs As Gs Cs As Gs As Cs Gs Gs As
moeGs moeAs moeCs moeG
4 A04 200 moeAs moeGs moeGs moeAs Gs As Cs Cs Cs Cs Gs As Cs Gs
moeAs moeAs moeCs moeG
5 A05 200 moeAs moeCs moeCs moeCs Cs Gs As Cs Gs As As Cs Gs As

```

moeCs moeTs moeGs moeG

6 A06 200 moeAs moeCs moeGs moeAs As Cs Gs As Cs Ts Gs Gs Cs Gs
moeAs moeCs moeAs moeG

7 A07 200 moeAs moeCs moeGs moeAs Cs Ts Gs Gs Cs Gs As Cs As Gs
5 moeGs moeTs moeAs moeG

8 A08 200 moeAs moeCs moeAs moeGs Gs Ts As Gs Gs Ts Cs Ts Ts Gs
moeGs moeTs moeGs moeG

9 A09 200 moeAs moeGs moeGs moeTs Cs Ts Ts Gs Gs Ts Gs Gs Gs Ts
moeGs moeAs moeCs moeG

10 10 A10 200 moeAs moeGs moeTs moeCs As Cs Gs As Cs As As Gs As As
moeAs moeCs moeAs moeC

11 A11 200 moeAs moeCs moeGs moeAs Cs As As Gs As As As Cs As Cs
moeGs moeGs moeTs moeC

12 A12 200 moeAs moeGs moeAs moeAs As Cs As Cs Gs Gs Ts Cs Gs Gs
15 moeTs moeCs moeCs moeT

13 B01 200 moeAs moeAs moeCs moeAs Cs Gs Gs Ts Cs Gs Gs Ts Cs Cs
moeTs moeGs moeTs moeC

14 B02 200 moeAs moeCs moeTs moeCs As Cs Ts Gs As Cs Gs Ts Gs Ts
moeCs moeTs moeCs moeA

20 15 B03 200 moeAs moeCs moeGs moeGs As As Gs Gs As As Cs Gs Cs Cs
moeAs moeCs moeTs moeT

16 B04 200 moeAs moeTs moeCs moeTs Gs Ts Gs Gs As Cs Cs Ts Ts Gs
moeTs moeCs moeTs moeC

17 B05 200 moeAs moeCs moeAs moeCs Ts Ts Cs Ts Ts Cs Cs Gs As Cs
25 moeCs moeGs moeTs moeG

18 B06 200 moeAs moeCs moeTs moeCs Ts Cs Gs As Cs As Cs As Gs Gs
moeAs moeCs moeGs moeT

19 B07 200 moeAs moeAs moeAs moeCs Cs Cs Cs As Gs Ts Ts Cs Gs Ts
moeCs moeTs moeAs moeA

30 20 B08 200 moeAs moeTs moeGs moeTs Cs Cs Cs Cs As As As Gs As Cs
moeTs moeAs moeTs moeG

- 21 B09 200 moeAs moeCs moeGs moeCs Ts Cs Gs Gs Gs As Cs Gs Gs Gs
moeTs moeCs moeAs moeG
- 22 B10 200 moeAs moeGs moeCs moeCs Gs As As Gs As As Gs As Gs Gs
moeTs moeTs moeAs moeC
- 5 23 B11 200 moeAs moeCs moeAs moeCs As Gs Ts As Gs As Cs Gs As As
moeAs moeGs moeCs moeT
- 24 B12 200 moeAs moeCs moeAs moeCs Ts Cs Ts Gs Gs Ts Ts Ts Cs Ts
moeGs moeGs moeAs moeC
- 25 C01 200 moeAs moeCs moeGs moeAs Cs Cs As Gs As As As Ts As Gs
10 moeTs moeTs moeTs moeT
- 26 C02 200 moeAs moeGs moeTs moeTs As As As As Gs Gs Gs Cs Ts Gs
moeCs moeTs moeAs moeG
- 27 C03 200 moeAs moeGs moeGs moeTs Ts Gs Ts Gs As Cs Gs As Cs Gs
moeAs moeGs moeGs moeT
- 15 28 C04 200 moeAs moeAs moeTs moeGs Ts As Cs Cs Ts As Cs Gs Gs Ts
moeTs moeGs moeGs moeC
- 29 C05 200 moeAs moeGs moeTs moeCs As Cs Gs Ts Cs Cs Ts Cs Ts Cs
moeTs moeGs moeTs moeC
- 30 C06 200 moeCs moeTs moeGs moeGs Cs Gs As Cs As Gs Gs Ts As Gs
20 moeGs moeTs moeCs moeT
- 31 C07 200 moeCs moeTs moeCs moeTs Gs Ts Gs Ts Gs As Cs Gs Gs Ts
moeGs moeGs moeTs moeC
- 32 C08 200 moeCs moeAs moeGs moeGs Ts Cs Gs Ts Cs Ts Ts Cs Cs Cs
moeGs moeTs moeGs moeG
- 25 33 C09 200 moeCs moeTs moeGs moeTs Gs Gs Ts As Gs As Cs Gs Ts Gs
moeGs moeAs moeCs moeA
- 34 C10 200 moeCs moeTs moeAs moeAs Cs Gs As Ts Gs Ts Cs Cs Cs Cs
moeAs moeAs moeAs moeG
- 35 C11 200 moeCs moeTs moeGs moeTs Ts Cs Gs As Cs As Cs Ts Cs Ts
30 moeGs moeGs moeTs moeT
- 36 C12 200 moeCs moeTs moeGs moeGs As Cs Cs As As Cs As Cs Gs Ts

moeTs moeGs moeTs moeC

37 D01 200 moeCs moeCs moeGs moeTs Cs Cs Gs Ts Gs Ts Ts Ts Gs Ts
moeTs moeCs moeTs moeG

38 D02 200 moeCs moeTs moeGs moeAs Cs Ts As Cs As As Cs As Gs As
5 moeCs moeAs moeCs moeC

39 D03 200 moeCs moeAs moeAs moeCs As Gs As Cs As Cs Cs As Gs Gs
moeGs moeGs moeTs moeC

40 D04 200 moeCs moeAs moeGs moeGs Gs Gs Ts Cs Cs Ts As Gs Cs Cs
moeGs moeAs moeCs moeT

10 41 D05 200 moeCs moeTs moeCs moeTs As Gs Ts Ts As As As As Gs Gs
moeGs moeCs moeTs moeG

42 D06 200 moeCs moeTs moeGs moeCs Ts As Gs As As Gs Gs As Cs Cs
moeGs moeAs moeGs moeG

43 D07 200 moeCs moeTs moeGs moeAs As As Ts Gs Ts As Cs Cs Ts As
15 moeCs moeGs moeGs moeT

44 D08 200 moeCs moeAs moeCs moeCs Cs Gs Ts Ts Ts Gs Ts Cs Cs Gs
moeTs moeCs moeAs moeA

45 D09 200 moeCs moeTs moeCs moeGs As Ts As Cs Gs Gs Gs Ts Cs As
moeGs moeTs moeCs moeA

20 46 D10 200 moeGs moeGs moeTs moeAs Gs Gs Ts Cs Ts Ts Gs Gs Ts Gs
moeGs moeGs moeTs moeG

47 D11 200 moeGs moeAs moeCs moeTs Ts Ts Gs Cs Cs Ts Ts As Cs Gs
moeGs moeAs moeAs moeG

48 D12 200 moeGs moeTs moeGs moeGs As Gs Ts Cs Ts Ts Ts Gs Ts Cs
25 moeTs moeGs moeTs moeG

49 E01 200 moeGs moeGs moeAs moeGs Ts Cs Ts Ts Ts Gs Ts Cs Ts Gs
moeTs moeGs moeGs moeT

50 E02 200 moeGs moeGs moeAs moeCs As Cs Ts Cs Ts Cs Gs As Cs As
moeCs moeAs moeGs moeG

30 51 E03 200 moeGs moeAs moeCs moeAs Cs As Gs Gs As Cs Gs Ts Gs Gs
moeCs moeGs moeAs moeG

- 52 E04 200 moeGs moeAs moeGs moeTs As Cs Gs As Gs Cs Gs Gs Gs Cs
moeCs moeGs moeAs moeA
- 53 E05 200 moeGs moeAs moeCs moeTs As Ts Gs Gs Ts As Gs As Cs Gs
moeCs moeTs moeCs moeG
- 5 54 E06 200 moeGs moeAs moeAs moeGs As Gs Gs Ts Ts As Cs As Cs As
moeGs moeTs moeAs moeG
- 55 E07 200 moeGs moeAs moeGs moeGs Ts Ts As Cs As Cs As Gs Ts As
moeGs moeAs moeCs moeG
- 56 E08 200 moeGs moeTs moeTs moeGs Ts Cs Cs Gs Ts Cs Cs Gs Ts Gs
10 moeTs moeTs moeTs moeG
- 57 E09 200 moeGs moeAs moeCs moeTs Cs Ts Cs Gs Gs Gs As Cs Cs As
moeCs moeCs moeAs moeC
- 58 E10 200 moeGs moeTs moeAs moeGs Gs As Gs As As Cs Cs As Cs Gs
moeAs moeCs moeCs moeA
- 15 59 E11 200 moeGs moeGs moeTs moeTs Cs Ts Ts Cs Gs Gs Ts Ts Gs Gs
moeTs moeTs moeAs moeT
- 60 E12 200 moeGs moeTs moeGs moeGs Gs Gs Ts Ts Cs Gs Ts Cs Cs Ts
moeTs moeGs moeGs moeG
- 61 F01 200 moeGs moeTs moeCs moeAs Cs Gs Ts Cs Cs Ts Cs Ts Gs As
20 moeAs moeAs moeTs moeG
- 62 F02 200 moeGs moeTs moeCs moeCs Ts Cs Cs Ts As Cs Cs Gs Ts Ts
moeTs moeCs moeTs moeC
- 63 F03 200 moeGs moeTs moeCs moeCs Cs Cs As Cs Gs Ts Cs Cs Gs Ts
moeCs moeTs moeTs moeC
- 25 64 F04 200 moeTs moeCs moeAs moeCs Cs As Gs Gs As Cs Gs Gs Cs Gs
moeGs moeAs moeCs moeC
- 65 F05 200 moeTs moeAs moeCs moeCs As As Gs Cs As Gs As Cs Gs Gs
moeAs moeGs moeAs moeC
- 66 F06 200 moeTs moeCs moeCs moeTs Gs Ts Cs Ts Ts Ts Gs As Cs Cs
30 moeAs moeCs moeTs moeC
- 67 F07 200 moeTs moeGs moeTs moeCs Ts Ts Ts Gs As Cs Cs As Cs Ts

moeCs moeAs moeCs moeT

68 F08 200 moeTs moeGs moeAs moeCs Cs As Cs Ts Cs As Cs Ts Gs As
moeCs moeGs moeTs moeG

69 F09 200 moeTs moeGs moeAs moeCs Gs Ts Gs Ts Cs Ts Cs As As Gs
5 moeTs moeGs moeAs moeC

70 F10 200 moeTs moeCs moeAs moeAs Gs Ts Gs As Cs Ts Ts Ts Gs Cs
moeCs moeTs moeTs moeA

71 F11 200 moeTs moeGs moeTs moeTs Ts As Ts Gs As Cs Gs Cs Ts Gs
moeGs moeGs moeGs moeT

10 72 F12 200 moeTs moeTs moeAs moeTs Gs As Cs Gs Cs Ts Gs Gs Gs Gs
moeTs moeTs moeGs moeG

73 G01 200 moeTs moeGs moeAs moeCs Gs Cs Ts Gs Gs Gs Gs Ts Ts Gs
moeGs moeAs moeTs moeC

74 G02 200 moeTs moeCs moeGs moeTs Cs Ts Ts Cs Cs Cs Gs Ts Gs Gs
15 moeAs moeGs moeTs moeC

75 G03 200 moeTs moeGs moeGs moeTs As Gs As Cs Gs Ts Gs Gs As Cs
moeAs moeCs moeTs moeT

76 G04 200 moeTs moeTs moeCs moeTs Ts Cs Cs Gs As Cs Cs Gs Ts Gs
moeAs moeCs moeAs moeT

20 77 G05 200 moeTs moeGs moeGs moeTs As Gs As Cs Gs Cs Ts Cs Gs Gs
moeGs moeAs moeCs moeG

78 G06 200 moeTs moeAs moeGs moeAs Cs Gs Cs Ts Cs Gs Gs Gs As Cs
moeGs moeGs moeGs moeT

79 G07 200 moeTs moeTs moeTs moeTs As Cs As Gs Ts Gs Gs Gs As As
25 moeCs moeCs moeTs moeG

80 G08 200 moeTs moeGs moeGs moeGs As As Cs Cs Ts Gs Ts Ts Cs Gs
moeAs moeCs moeAs moeC

81 G09 200 moeTs moeCs moeGs moeGs Gs As Cs Cs As Cs Cs As Cs Ts
moeAs moeGs moeGs moeG

30 82 G10 200 moeTs moeAs moeGs moeGs As Cs As As As Cs Gs Gs Ts As
moeGs moeGs moeAs moeG

83 G11 200 moeTs moeGs moeCs moeTs As Gs As As Gs Gs As Cs Cs Gs
moeAs moeGs moeGs moeT

84 G12 200 moeTs moeCs moeTs moeGs Ts Cs As Cs Ts Cs Cs Gs As Cs
moeGs moeTs moeGs moeG

5 Reagent file (.tab File)

Table 5 is a .tab file for reagents necessary for synthesizing an oligonucleotides having both 2'-O-(2-methoxyethyl)nucleosides and 2'-deoxy nucleosides located therein.

Table 5

Identity of columns: **GroupName, Bottle ID, ReagentName, FlowRate, Concentration.**

- 10 Wherein reagent name is identified using base identifier, “moe” indicated a 2'-O-(2-methoxyethyl) substituted nucleoside and “cpg” indicates a control pore glass solid support medium. The columns wrap around to next line when longer than one line.

SUPPORT

BEGIN

- 15 0 moeG moeG cpg 100 1
0 moe5meC moe5meC cpg 100 1
0 moeA moeA cpg 100 1
0 moeT moeT cpg 100 1
END

20 DEBLOCK

BEGIN

70 TCA TCA 100 1
END

WASH

- 25 BEGIN
65 ACN ACN 190 1

END

OXIDIZERS

BEGIN

68 BEAU BEAUCAGE 320 1

5 END

CAPPING

BEGIN

66 CAP_B CAP_B 220 1

67 CAP_A CAP_A 230 1

10 END

DEOXY THIOATE

BEGIN

31,32 Gs deoxyG 270 1

39,40 5meCs 5methyldeoxyC 270 1

15 37,38 As deoxyA 270 1

29,30 Ts deoxyT 270 1

END

MOE-THIOATE

20 BEGIN

15,16 moeGs methoxyethoxyG 240 1

23,24 moe5meCs methoxyethoxyC 240 1

21,22 moeAs methoxyethoxyA 240 1

13,14 moeTs methoxyethoxyT 240 1

25 END

ACTIVATORS

BEGIN

5,6,7,8 SET s-ethyl-tet 280

Activates

DEOXY_THIOATE

MOE_THIOATE

END

5 **EXAMPLE 4: Oligonucleotide Synthesis - 96 Well Plate Format**

10 Oligonucleotides were synthesized via solid phase P(III) phosphoramidite chemistry using a multi well automated synthesizer utilizing input files as described in EXAMPLE 3 above. The oligonucleotides were synthesized by assembling 96 sequences simultaneously in a standard 96 well format. Phosphodiester internucleotide linkages were afforded by oxidation with aqueous iodine. Phosphorothioate internucleotide linkages were generated by sulfurization utilizing 3H-1,2 benzodithiole-3-one 1,1 dioxide (Beaucage Reagent) in anhydrous acetonitrile. Standard base-protected beta-cyanoethyl-diisopropyl phosphoramidites were purchased from commercial vendors (e.g. PE/ABI, Pharmacia). Non-standard nucleosides are synthesized as per known literature or patented methods. They are utilized as base protected beta-cyanoethyl-diisopropyl phosphoramidites.

15 Following synthesis, oligonucleotides were cleaved from support and deprotected with concentrated NH_4OH at elevated temperature ($55\text{-}60^\circ\text{C}$) for 12-16 hours and the released product then dried *in vacuo*. The dried product was then re-suspended in sterile water to afford a master plate from which all analytical and test plate samples are then diluted utilizing robotic pipettors.

20 **EXAMPLE 5: Alternative Oligonucleotide Synthesis**

Unsubstituted and substituted phosphodiester oligonucleotides are alternately synthesized on an automated DNA synthesizer (Applied Biosystems model 380B) using standard phosphoramidite chemistry with oxidation by iodine.

25 Phosphorothioates are synthesized as per the phosphodiester oligonucleotides except the standard oxidation bottle was replaced by 0.2 M solution of 3H-1,2-benzodithiole-3-one 1,1-dioxide in acetonitrile for the stepwise thiation of the phosphite linkages. The thiation wait step was increased to 68 sec and was followed by the capping

step. After cleavage from the CPG column and deblocking in concentrated ammonium hydroxide at 55°C (18 hr), the oligonucleotides were purified by precipitating twice with 2.5 volumes of ethanol from a 0.5 M NaCl solution.

Phosphinate oligonucleotides are prepared as described in U.S. Patent 5,508,270,
5 incorporated herein by reference in its entirety.

Alkyl phosphonate oligonucleotides are prepared as described in U.S. Patent 4,469,863, incorporated herein by reference in its entirety.

3'-Deoxy-3'-methylene phosphonate oligonucleotides are prepared as described in U.S. Patents 5,610,289 or 5,625,050, each of which is incorporated herein by reference in
10 its entirety.

Phosphoramidite oligonucleotides are prepared as described in U.S. Patent, 5,256,775 or U.S. Patent 5,366,878, incorporated herein by reference in its entirety.

Alkylphosphonothioate oligonucleotides are prepared as described in published PCT applications PCT/US94/00902 and PCT/US93/06976 (published as WO 94/17093
15 and WO 94/02499, respectively), each of which is incorporated herein by reference in its entirety.

3'-Deoxy-3'-amino phosphoramidate oligonucleotides are prepared as described in U.S. Patent 5,476,925, incorporated herein by reference in its entirety.

Phosphotriester oligonucleotides are prepared as described in U.S. Patent
20 5,023,243, incorporated herein by reference in its entirety.

Boranophosphate oligonucleotides are prepared as described in U.S. Patents 5,130,302 and 5,177,198, each of which is incorporated herein by reference in its entirety.

Methylenemethylimino linked oligonucleosides, also identified as MMI linked oligonucleosides, methylenedimethylhydrazo linked oligonucleosides, also identified as
25 MDH linked oligonucleosides, and methylenecarbonylamino linked oligonucleosides, also identified as amide-3 linked oligonucleosides, and methyleneaminocarbonyl linked oligonucleosides, also identified as amide-4 linked oligonucleosides, as well as mixed backbone compounds having, for instance, alternating MMI and PO or PS linkages are prepared as described in U.S. Patents 5,378,825; 5,386,023; 5,489,677; 5,602,240 and 5,610,289, each
30 of which is incorporated herein by reference in its entirety.

Formacetal and thioformacetal linked oligonucleosides are prepared as described in

U.S. Patents 5,264,562 and 5,264,564, each of which is incorporated herein by reference in its entirety.

Ethylene oxide linked oligonucleosides are prepared as described in U.S. Patent 5,223,618, incorporated herein by reference in its entirety.

5 **EXAMPLE 6: PNA Synthesis**

Peptide nucleic acids (PNAs) are prepared in accordance with any of the various procedures referred to in Peptide Nucleic Acids (PNA): Synthesis, Properties and Potential Applications, *Bioorganic & Medicinal Chemistry*, 1996, 4, 5. They may also be prepared in accordance with U.S. Patents 5,539,082; 5,700,922, and 5,719,262, each of which is
10 incorporated herein by reference in its entirety.

EXAMPLE 7: Chimeric Oligonucleotide Synthesis

Chimeric oligonucleotides, oligonucleosides or mixed oligonucleotides/oligonucleosides of the invention can be of several different types. These include a first type wherein the "gap" segment of linked nucleosides is positioned between 5' and 3' "wing" segments of linked nucleosides and a second "open end" type wherein the
15 "gap" segment is located at either the 3' or the 5' terminus of the oligomeric compound. Oligonucleotides of the first type are also known in the art as "gapmers" or gapped oligonucleotides. Oligonucleotides of the second type are also known in the art as "hemimers" or "wingmers."

20 **A. [2'-O-Me]--[2'-deoxy]--[2'-O-Me] Chimeric Phosphorothioate Oligonucleotides**

Chimeric oligonucleotides having 2'-O-alkyl phosphorothioate and 2'-deoxy phosphorothioate oligonucleotide segments are synthesized using 2'-deoxy-5'-dimethoxytrityl-3'-O-phosphoramidites for the DNA portion and 5'-dimethoxytrityl-2'-O-methyl-3'-O-phosphoramidites for 5' and 3' wings. The standard synthesis cycle is
25 modified by increasing the wait step after the delivery of tetrazole and base to 600 s repeated four times for DNA and twice for 2'-O-methyl. The fully protected oligonucleotide was cleaved from the support and the phosphate group is deprotected in 3:1 Ammonia/Ethanol at room temperature overnight then lyophilized to dryness.

Treatment in methanolic ammonia for 24 hrs at room temperature is done to deprotect all bases and the samples are again lyophilized to dryness.

B. [2'-O-(2-Methoxyethyl)]--[2'-deoxy]--[2'-O-(2-Methoxyethyl)]

Chimeric Phosphorothioate Oligonucleotides

5 [2'-O-(2-methoxyethyl)]--[2'-deoxy]--[2'-O-(2-methoxyethyl)] chimeric phosphorothioate oligonucleotides are prepared as per the procedure above for the 2'-O-methyl chimeric oligonucleotide, with the substitution of 2'-O-(2-methoxyethyl) amidites for the 2'-O-methyl amidites.

C. [2'-O-(2-Methoxyethyl)Phosphodiester]--[2'-deoxy Phosphorothioate]--[2'-O-(2-Methoxyethyl) Phosphodiester] Chimeric Oligonucleotide

10 [2'-O-(2-methoxyethyl phosphodiester)]--[2'-deoxy phosphorothioate]--[2'-O-(2-methoxyethyl) phosphodiester] chimeric oligonucleotides are prepared as per the above procedure for the 2'-O-methyl chimeric oligonucleotide with the substitution of 2'-O-(2-methoxyethyl) amidites for the 2'-O-methyl amidites in the wing portions. Sulfurization utilizing 3,4,5-trimethyl-1,2,4-benzodithiolane-3-one 1,1 dioxides (Beaucage Reagent) is used to generate the phosphorothioate internucleotide linkages within the wing portions of the chimeric structures. Oxidization with iodine is used to generate the phosphodiester internucleotide linkages for the center gap.

15 Other chimeric oligonucleotides, chimeric oligonucleosides and mixed chimeric oligonucleotides/oligonucleosides are synthesized according to United States Patent 5,623,065, which is incorporated herein by reference in its entirety.

EXAMPLE 8: Output Oligonucleotides From Automated Oligonucleotide Synthesis

Using the .seq files, the .cmd files and .tab file of Example 3, oligonucleotides were prepared as per the protocol of the 96 well format of Example 4. The
25 oligonucleotides were prepared utilizing phosphorothioate chemistry to give in one instance a first library of phosphorothioate oligodeoxynucleotides. The oligonucleotides were prepared in a second instance as a second library of hybrid oligonucleotides having phosphorothioate backbones with a first and third "wing" region of 2'-O-(2-methoxyethyl)nucleotides on either side of a center gap region of 2'-deoxy nucleotides.
30 The two libraries contained the same set of oligonucleotide sequences. Thus the two

libraries are redundant with respect to sequence but are unique with respect to the combination of sequence and chemistry. Because the sequences of the second library of compounds is the same as the first (however the chemistry is different), for brevity sake, the second library is not shown.

- 5 For illustrative purposes Tables 6-a and 6-b show the sequences of an initial first library, i.e., a library of phosphorothioate oligonucleotides targeted to a CD40 target. The compounds of Table 6-a shows the members of this library listed in compliance with the established rule for listing SEQ ID NO:, i.e., in numerical SEQ ID NO: order.

Table 6-a

10 **Sequences of Oligonucleotides Targeted to CD40 by SEQ ID NO.:**

	NUCLEOBASE SEQUENCE	SEQ ID NO.
	CCAGGCGGCAGGACCACT	1
	GACCAGGCGGCAGGACCA	2
	AGGTGAGACCAGGCGGCA	3
15	CAGAGGCAGACGAACCAT	4
	GCAGAGGCAGACGAACCA	5
	GCAAGCAGCCCCAGAGGA	6
	GGTCAGCAAGCAGCCCCA	7
	GACAGCGGTCAGCAAGCA	8
20	GATGGACAGCGGTCAGCA	9
	TCTGGATGGACAGCGGTC	10
	GGTGGTTCTGGATGGACA	11
	GTGGGTGGTTCTGGATGG	12
	GCAGTGGGTGGTTCTGGA	13
25	CACAAAGAACAGCACTGA	14
	CTGGCACAAAGAACAGCA	15
	TCCTGGCTGGCACAAAGA	16
	CTGTCCTGGCTGGCACAA	17
	CTCACCAGTTTCTGTCCT	18
30	TCACTCACCAGTTTCTGT	19

	GTGCAGTCACTCACCAGT	20
	ACTCTGTGCAGTCACTCA	21
	CAGTGAAGTCTGTGCAGT	22
	ATTCCGTTTCAGTGAAGT	23
5	GAAGGCATTCCGTTTCAG	24
	TTCACCGCAAGGAAGGCA	25
	CTCTGTTCCAGGTGTCTA	26
	CTGGTGGCAGTGTGTCTC	27
	TGGGGTCGCAGTATTTGT	28
10	GGTTGGGGTCGCAGTATT	29
	CTAGGTGGGGTCGCAGT	30
	GGTGCCCTTCTGCTGGAC	31
	CTGAGGTGCCCTTCTGCT	32
	GTGTCTGTTTCTGAGGTG	33
15	TGGTGTCTGTTTCTGAGG	34
	ACAGGTGCAGATGGTGTG	35
	TTCACAGGTGCAGATGGT	36
	GTGCCAGCCTTCTTCACA	37
	TACAGTGCCAGCCTTCTT	38
20	GGACACAGCTCTCACAGG	39
	TGCAGGACACAGCTCTCA	40
	GAGCGGTGCAGGACACAG	41
	AAGCCGGGCGAGCATGAG	42
	AATCTGCTTGACCCCAA	43
25	GAAACCCCTGTAGCAATC	44
	GTATCAGAAACCCCTGTA	45
	GCTCGCAGATGGTATCAG	46
	GCAGGGCTCGCAGATGGT	47
	TGGGCAGGGCTCGCAGAT	48
30	GACTGGGCAGGGCTCGCA	49
	CATTGGAGAAGAAGCCGA	50

	GATGACACATTGGAGAAG	51
	GCAGATGACACATTGGAG	52
	TCGAAAGCAGATGACACA	53
	GTCCAAGGGTGACATTTT	54
5	CACAGCTTGTCCAAGGGT	55
	TTGGTCTCACAGCTTGTC	56
	CAGGTCTTTGGTCTCACA	57
	CTGTTGCACAACCAGGTC	58
	GTTTGTGCCTGCCTGTTG	59
10	GTCTTGTTTGTGCCTGCC	60
	CCACAGACAACATCAGTC	61
	CTGGGGACCACAGACAAC	62
	TCAGCCGATCCTGGGGAC	63
	CACCACCAGGGCTCTCAG	64
15	GGGATCACCACCAGGGCT	65
	GAGGATGGCAAACAGGAT	66
	ACCAGCACCAAGAGGATG	67
	TTTTGATAAAGACCAGCA	68
	TATTGGTTGGCTTCTTGG	69
20	GGGTTCCCTGCTTGGGGTG	70
	GTCGGGAAAATTGATCTC	71
	GATCGTCGGGAAAATTGA	72
	GGAGCCAGGAAGATCGTC	73
	TGGAGCCAGGAAGATCGT	74
25	TGGAGCAGCAGTGTTGGA	75
	GTAAAGTCTCCTGCACTG	76
	TGGCATCCATGTAAAGTC	77
	CGGTTGGCATCCATGTAA	78
	CTCTTTGCCATCCTCCTG	79
30	CTGTCTCTCCTGCACTGA	80
	GGTGCAGCCTCACTGTCT	81

AACTGCCTGTTTGCCAC	82
CTTCTGCCTGCACCCCTG	83
ACTGACTGGGCATAGCTC	84

5 The sequences shown in Table 6-a, above, and Table 6-b, below, are in a 5' to 3' direction. This is reversed with respect to 3' to 5' direction shown in the .seq files of Example 3. For synthesis purposes, the .seq files are generated reading from 3' to 5'. This allows for aligning all of the 3' most "A" nucleosides together, all of the 3' most "G" nucleosides together, all of the 3' most "C" nucleosides together and all of the 3' most "T" nucleosides together. Thus when the first nucleoside of each particular oligonucleotide (attached to the solid support) is added to the wells on the plates, machine movement is reduced since an automatic pipette can move in a linear manner down one row and up another on the 96 well plate.

15 The location of the well holding each particular oligonucleotides is indicated by row and column. There are eight rows designated A to H and twelve columns designated 1 to 12 in a typical 96 well format plate. Any particular well location is indicated by its "Well No." which is indicated by the combination of the row and the column, e.g. A08 is the well at row A, column 8.

20 In Table 6-b below, the oligonucleotides of Table 6-a are shown reordered according to the Well No. on their synthesis plate. The order shown in Table 6-b is the actually order as synthesized on an automated synthesizer taking advantage of the preferred placement of the first nucleoside according to the above alignment criteria.

Table 6-b:

25 **Sequences of Oligonucleotides Targeted to CD40 Order by Synthesis Well No.**

Well No.		SEQ ID NO:
A01	GACCAGGCGGCAGGACCA	2
A02	AGGTGAGACCAGGCGGCA	3
A03	GCAGAGGCAGACGAACCA	5
A04	GCAAGCAGCCCCAGAGGA	6
A05	GGTCAGCAAGCAGCCCCA	7
A06	GACAGCGGTCAGCAAGCA	8
A07	GATGGACAGCGGTCAGCA	9
A08	GGTGGTTCTGGATGGACA	11

	A09	GCAGTGGGTGGTTCTGGA	13
	A10	CACAAAGAACAGCACTGA	14
	A11	CTGGCACAAAGAACAGCA	15
	A12	TCCTGGCTGGCACAAAGA	16
5	B01	CTGTCCTGGCTGGCACAA	17
	B02	ACTCTGTGCAGTCACTCA	21
	B03	TTCACCGCAAGGAAGGCA	25
	B04	CTCTGTTCCAGGTGTCTA	26
	B05	GTGCCAGCCTTCTTCACA	37
10	B06	TGCAGGACACAGCTCTCA	40
	B07	AATCTGCTTGACCCCAA	43
	B08	GTATCAGAAACCCCTGTA	45
	B09	GACTGGGCAGGGCTCGCA	49
	B10	CATTGGAGAAGAAGCCGA	50
15	B11	TCGAAAGCAGATGACACA	53
	B12	CAGGTCTTTGGTCTCACA	57
	C01	TTTTGATAAAGACCAGCA	68
	C02	GATCGTCGGGAAAATTGA	72
	C03	TGGAGCAGCAGTGTTGGA	75
20	C04	CGGTTGGCATCCATGTAA	78
	C05	CTGTCTCTCCTGCACTGA	80
	C06	TCTGGATGGACAGCGGTC	10
	C07	CTGGTGGCAGTGTGTCTC	27
	C08	GGTGCCCTTCTGCTGGAC	31
25	C09	ACAGGTGCAGATGGTGTC	35
	C10	GAAACCCCTGTAGCAATC	44
	C11	TTGGTCTCACAGCTTGTC	56
	C12	CTGTTGCACAACCAGGTC	58
	D01	GTCTTGTTTGTGCCTGCC	60
30	D02	CCACAGACAACATCAGTC	61
	D03	CTGGGGACCACAGACAAC	62
	D04	TCAGCCGATCCTGGGGAC	63
	D05	GTCGGGAAAATTGATCTC	71
	D06	GGAGCCAGGAAGATCGTC	73
35	D07	TGGCATCCATGTAAAGTC	77
	D08	AACTGCCTGTTTGCCAC	82
	D09	ACTGACTGGGCATAGCTC	84
	D10	GTGGGTGGTTCTGGATGG	12
	D11	GAAGGCATTCCGTTTCAG	24
40	D12	GTGTCTGTTTCTGAGGTG	33
	E01	TGGTGTCTGTTTCTGAGG	34
	E02	GGACACAGCTCTCACAGG	39
	E03	GAGCGGTGCAGGACACAG	41
	E04	AAGCCGGGCGAGCATGAG	42
45	E05	GCTCGCAGATGGTATCAG	46
	E06	GATGACACATTGGAGAAG	51
	E07	GCAGATGACACATTGGAG	52
	E08	GTTTGTGCCTGCCTGTTG	59
	E09	CACCACCAGGGCTCTCAG	64
50	E10	ACCAGCACCAAGAGGATG	67
	E11	TATTGGTTGGCTTCTTGG	69

5	E12	GGGTTCTGCTTGGGGTG	70
	F01	GTAAAGTCTCCTGCACTG	76
	F02	CTCTTTGCCATCCTCCTG	79
	F03	CTTCTGCCTGCACCCCTG	83
	F04	CCAGGCGGCAGGACCACT	1
10	F05	CAGAGGCAGACGAACCAT	4
	F06	CTCACCAGTTTCTGTCCT	18
	F07	TCACTCACCAGTTTCTGT	19
	F08	GTGCAGTCACTCACCAGT	20
	F09	CAGTGAACCTCTGTGCAGT	22
15	F10	ATTCCGTTTCAGTGAACCT	23
	F11	TGGGGTCGCAGTATTTGT	28
	F12	GGTTGGGGTCGCAGTATT	29
	G01	CTAGGTTGGGGTCGCAGT	30
	G02	CTGAGGTGCCCTTCTGCT	32
20	G03	TTCACAGGTGCAGATGGT	36
	G04	TACAGTGCCAGCCTTCTT	38
	G05	GCAGGGCTCGCAGATGGT	47
	G06	TGGGCAGGGCTCGCAGAT	48
	G07	GTCCAAGGGTGACATTTT	54
25	G08	CACAGCTTGTCCAAGGGT	55
	G09	GGGATCACCACCAGGGCT	65
	G10	GAGGATGGCAAACAGGAT	66
	G11	TGGAGCCAGGAAGATCGT	74
	G12	GGTGCAGCCTCACTGTCT	81

EXAMPLE 9: Oligonucleotide Analysis

A. Oligonucleotide Analysis - 96 Well Plate Format

The concentration of oligonucleotide in each well was assessed by dilution of samples and UV absorption spectroscopy. The full-length integrity of the individual products was evaluated by capillary electrophoresis (CE) in either the 96 well format (Beckman MDQ) or, for individually prepared samples, on a commercial CE apparatus (e.g., Beckman 5000, ABI 270). Base and backbone composition was confirmed by mass analysis of the compounds utilizing electrospray-mass spectroscopy. All assay test plates were diluted from the master plate using single and multi-channel robotic pipettors.

B. Alternative Oligonucleotide Analysis

After cleavage from the controlled pore glass support (Applied Biosystems) and deblocking in concentrated ammonium hydroxide at 55°C for 18 hours, the oligonucleotides or oligonucleosides are purified by precipitation twice out of 0.5 M NaCl with 2.5 volumes ethanol. Synthesized oligonucleotides are analyzed by polyacrylamide gel electrophoresis on denaturing gels. Oligonucleotide purity is checked by ³¹P nuclear

magnetic resonance spectroscopy, and/or by HPLC, as described by Chiang *et al.*, *J. Biol. Chem.* 1991, 266, 18162.

EXAMPLE 10: Automated Assay of CD40 Oligonucleotide Activity

5 A. Poly(A)+ mRNA isolation.

Poly(A)+ mRNA was isolated according to Miura *et al.* (*Clin. Chem.*, 1996, 42, 1758). Briefly, for cells grown on 96-well plates, growth medium was removed from the cells and each well was washed with 200 μ l cold PBS. 60 μ l lysis buffer (10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 0.5 M NaCl, 0.5% NP-40, 20 mM vanadyl-ribonucleoside complex) was added to each well, the plate was gently agitated and then incubated at room temperature for five minutes. 55 μ l of lysate was transferred to Oligo d(T) coated 96 well plates (AGCT Inc., Irvine, CA). Plates were incubated for 60 minutes at room temperature, washed 3 times with 200 ml of wash buffer (10 mM Tris-HCl pH 7.6, 1 mM EDTA, 0.3 M NaCl). After the final wash, the plate was blotted on paper towels to remove excess wash buffer and then air-dried for 5 minutes. 60 ml of elution buffer (5 mM Tris-HCl pH 7.6), preheated to 70°C was added to each well, the plate was incubated on a 90°C plate for 5 minutes, and the eluate then transferred to a fresh 96-well plate. Cells grown on 100 mm or other standard plates may be treated similarly, using appropriate volumes of all solutions.

20 B. Total RNA isolation

Total mRNA was isolated using an RNEASY 96 $\hat{\circ}$ kit and buffers purchased from Qiagen Inc. (Valencia CA) following the manufacturer's recommended procedures. Briefly, for cells grown on 96-well plates, growth medium was removed from the cells and each well was washed with 200 mL cold PBS. 100 mL Buffer RLT was added to each well and the plate vigorously agitated for 20 seconds. 100 mL of 70% ethanol was then added to each well and the contents mixed by pipetting three times up and down. The samples were then transferred to the RNEASY 96 $\hat{\circ}$ well plate attached to a QIAVAC $\hat{\circ}$ manifold fitted with a waste collection tray and attached to a vacuum source. Vacuum was applied for 15 seconds. 1 mL of Buffer RW1 was added to each well of the RNEASY 96 $\hat{\circ}$ plate and the vacuum again applied for 15 seconds. 1 mL of Buffer RPE was then added to each well of the RNEASY 96 $\hat{\circ}$ plate and the vacuum applied for a period of 15 seconds. The

Buffer RPE wash was then repeated and the vacuum was applied for an additional 10 minutes. The plate was then removed from the QIAVACÔ manifold and blotted dry on paper towels. The plate was then re-attached to the QIAVACÔ manifold fitted with a collection tube rack containing 1.2 mL collection tubes. RNA was then eluted by
5 pipetting 60 mL water into each well, incubating 1 minute, and then applying the vacuum for 30 seconds. The elution step was repeated with an additional 60 mL water.

C. RT-PCR Analysis of CD40 mRNA Levels

Quantitation of CD40 mRNA levels was determined by reverse transcriptase
10 polymerase chain reaction (RT-PCR) using the ABI PRISM™_7700 Sequence Detection System (PE-Applied Biosystems, Foster City, CA) according to manufacturer's instructions. This is a closed-tube, non-gel-based, fluorescence detection system which allows high-throughput quantitation of polymerase chain reaction (PCR) products in real-time.

15 As opposed to standard PCR, in which amplification products are quantitated after the PCR is completed, products in RT-PCR are quantitated as they accumulate. This is accomplished by including in the PCR reaction an oligonucleotide probe that anneals specifically between the forward and reverse PCR primers, and contains two fluorescent dyes. A reporter dye (e.g., JOE or FAM, PE-Applied Biosystems, Foster City, CA) is
20 attached to the 5' end of the probe and a quencher dye (e.g., TAMRA, PE-Applied Biosystems, Foster City, CA) is attached to the 3' end of the probe. When the probe and dyes are intact, reporter dye emission is quenched by the proximity of the 3' quencher dye. During amplification, annealing of the probe to the target sequence creates a substrate that can be cleaved by the 5'-exonuclease activity of Taq polymerase. During the extension
25 phase of the PCR amplification cycle, cleavage of the probe by Taq polymerase releases the reporter dye from the remainder of the probe (and hence from the quencher moiety) and a sequence-specific fluorescent signal is generated.

With each cycle, additional reporter dye molecules are cleaved from their respective probes, and the fluorescence intensity is monitored at regular (six-second)
30 intervals by laser optics built into the ABI PRISM™ 7700 Sequence Detection System. In each assay, a series of parallel reactions containing serial dilutions of mRNA from

untreated control samples generates a standard curve that is used to quantitate the percent inhibition after antisense oligonucleotide treatment of test samples.

RT-PCR reagents were obtained from PE-Applied Biosystems, Foster City, CA. RT-PCR reactions were carried out by adding 25 ml PCR cocktail (1x TAQMAN™ buffer A, 5.5 mM MgCl₂, 300 mM each of dATP, dCTP and dGTP, 600 mM of dUTP, 100 nM
5 each of forward primer, reverse primer, and probe, 20 U RNase inhibitor, 1.25 units AMPLITAQ GOLD™, and 12.5 U MuLV reverse transcriptase) to 96 well plates containing 25 ml poly(A) mRNA solution. The RT reaction was carried out by incubation for 30 minutes at 48°C. following a 10 minute incubation at 95°C to activate the
10 AMPLITAQ GOLD™, 40 cycles of a two-step PCR protocol were carried out: 95°C for 15 seconds (denaturation) followed by 60°C for 1.5 minutes (annealing/extension).

For CD40, the PCR primers were:

forward: 5' CAGAGTTCACTGAAACGGAATGC 3'
(SEQ ID NO:86)
15 reverse: 5' GGTGGCAGTGTGTCTCTCTGTTC 3' (SEQ ID NO:87), and
PCR probe: 5' FAM-TTCCTTGCGGTGAAAGCGAATTCCT-TAMRA 3' (SEQ ID NO:88) where FAM (PE-Applied Biosystems, Foster City, CA) is the fluorescent reporter dye and TAMRA (PE-Applied Biosystems, Foster City, CA) is the quencher dye.

For GAPDH, the PCR primers were:

20 forward: 5' GAAGGTGAAGGTCGGAGTC 3' (SEQ ID NO:89)
reverse: 5' GAAGATGGTGATGGGATTTC 3' (SEQ ID NO:90), and
PCR probe: 5' JOE-CAAGCTTCCCGTTCTCAGCC-TAMRA 3' (SEQ ID NO. 91)
where JOE (PE-Applied Biosystems, Foster City, CA) is the fluorescent reporter dye and
TAMRA (PE-Applied Biosystems, Foster City, CA) is the quencher dye.

25

EXAMPLE 11: Inhibition of CD40 Expression by Phosphorothioate Oligodeoxynucleotides

In accordance with the present invention, a series of oligonucleotides complementary to mRNA were designed to target different regions of the human CD40
30 mRNA, using published sequences (GenBank accession number X60592, incorporated herein by reference as SEQ ID NO: 85). The oligonucleotides are shown in Table 7.

Target sites are indicated by the beginning nucleotide numbers, as given in the sequence source reference (X60592), to which the oligonucleotide binds. All compounds in Table 7 are oligodeoxynucleotides with phosphorothioate backbones (internucleoside linkages) throughout. Data are averages from three experiments.

5

Table 7:

Inhibition of CD40 mRNA Levels by Phosphorothioate Oligodeoxynucleotides

TARGET		SEQ ID			
ISIS#	SITE	SEQUENCE	% INHIB.	NO.	
10	18623	18	CCAGGCGGCAGGACCA	30.71	1
	18624	20	GACCAGGCGGCAGGAC	28.09	2
	18625	26	AGGTGAGACCAGGCGG	21.89	3
	18626	48	CAGAGGCAGACGAACC	0.00	4
	18627	49	GCAGAGGCAGACGAAC	0.00	5
	18628	73	GCAAGCAGCCCCAGAG	0.00	6
15	18629	78	GGTCAGCAAGCAGCCC	29.96	7
	18630	84	GACAGCGGTCAGCAAG	0.00	8
	18631	88	GATGGACAGCGGTCAG	0.00	9
	18632	92	TCTGGATGGACAGCGG	0.00	10
	18633	98	GGTGGTTCTGGATGGA	0.00	11
	18634	101	GTGGGTGGTTCTGGAT	0.00	12
20	18635	104	GCAGTGGGTGGTTCTG	0.00	13
	18636	152	CACAAAGAACAGCACT	0.00	14
	18637	156	CTGGCACAAAGAACAG	0.00	15
	18638	162	TCCTGGCTGGCACAAA	0.00	16
	18639	165	CTGTCTGGCTGGCAC	4.99	17
	18640	176	CTCACCAGTTTCTGTCC	0.00	18
25	18641	179	TCACTCACCAGTTTCTG	0.00	19
	18642	185	GTGCAGTCACTCACCA	0.00	20
	18643	190	ACTCTGTGCAGTCACTC	0.00	21
	18644	196	CAGTGA ACTCTGTGCA	5.30	22
	18645	205	ATTCCGTTTCAGTGAAC	0.00	23
	18646	211	GAAGGCATTCCGTTTC	9.00	24
30	18647	222	TTCACCGCAAGGAAGG	0.00	25
	18648	250	CTCTGTTCCAGGTGTCT	0.00	26
	18649	267	CTGGTGGCAGTGTGTC	0.00	27
	18650	286	TGGGGTTCGCAGTATTT	0.00	28
	18651	289	GGTTGGGGTCGCAGTA	0.00	29
	18652	292	CTAGGTTGGGGTCGCA	0.00	30
35	18653	318	GGTGCCCTTCTGCTGG	19.67	31
	18654	322	CTGAGGTGCCCTTCTGC	15.63	32
	18655	332	GTGTCTGTTTCTGAGGT	0.00	33
	18656	334	TGGTGTCTGTTTCTGAG	0.00	34
	18657	345	ACAGGTGCAGATGGTG	0.00	35
	18658	348	TTCACAGGTGCAGATG	0.00	36

	18659	360	GTGCCAGCCTTCTTCAC	5.67	37
	18660	364	TACAGTGCCAGCCTTCT	7.80	38
	18661	391	GGACACAGCTCTCACA	0.00	39
	18662	395	TGCAGGACACAGCTCT	0.00	40
5	18663	401	GAGCGGTGCAGGACAC	0.00	41
	18664	416	AAGCCGGGCGAGCATG	0.00	42
	18665	432	AATCTGCTTGACCCCA	5.59	43
	18666	446	GAAACCCCTGTAGCAA	0.10	44
	18667	452	GTATCAGAAACCCCTG	0.00	45
10	18668	463	GCTCGCAGATGGTATC	0.00	46
	18669	468	GCAGGGCTCGCAGATG	34.05	47
	18670	471	TGGGCAGGGCTCGCAG	0.00	48
	18671	474	GACTGGGCAGGGCTCG	2.71	49
	18672	490	CATTGGAGAAGAAGCC	0.00	50
15	18673	497	GATGACACATTGGAGA	0.00	51
	18674	500	GCAGATGACACATTGG	0.00	52
	18675	506	TCGAAAGCAGATGACA	0.00	53
	18676	524	GTCCAAGGGTGACATT	8.01	54
	18677	532	CACAGCTTGTTCCAAGG	0.00	55
20	18678	539	TTGGTCTCACAGCTTGT	0.00	56
	18679	546	CAGGTCTTTGGTCTCAC	6.98	57
	18680	558	CTGTTGCACAACCAGG	18.76	58
	18681	570	GTTTGTGCCTGCCTGTT	2.43	59
	18682	575	GTCTTGTTTGTGCCTGC	0.00	60
25	18683	590	CCACAGACAACATCAG	0.00	61
	18684	597	CTGGGGACCACAGACA	0.00	62
	18685	607	TCAGCCGATCCTGGGG	0.00	63
	18686	621	CACCACCAGGGCTCTC	23.31	64
	18687	626	GGGATCACCACCAGGG	0.00	65
30	18688	657	GAGGATGGCAAACAGG	0.00	66
	18689	668	ACCAGCACCAAGAGGA	0.00	67
	18690	679	TTTTGATAAAGACCAG	0.00	68
	18691	703	TATTGGTTGGCTTCTTG	0.00	69
	18692	729	GGGTTCTTGCTTGGGG	0.00	70
35	18693	750	GTCGGGAAAATTGATC	0.00	71
	18694	754	GATCGTCGGGAAAATT	0.00	72
	18695	765	GGAGCCAGGAAGATCG	0.00	73
	18696	766	TGGAGCCAGGAAGATC	0.00	74
	18697	780	TGGAGCAGCAGTGTTG	0.00	75
40	18698	796	GTAAAGTCTCCTGCAC	0.00	76
	18699	806	TGGCATCCATGTAAAG	0.00	77
	18700	810	CGGTTGGCATCCATGT	0.00	78
	18701	834	CTCTTTGCCATCCTCCT	4.38	79
	18702	861	CTGTCTCTCCTGCACTG	0.00	80
45	18703	873	GGTGCAGCCTCACTGT	0.00	81
	18704	910	AACTGCCTGTTTGCCCA	33.89	82
	18705	954	CTTCTGCCTGCACCCCT	0.00	83
	18706	976	ACTGACTGGGCATAGC	0.00	84

As shown in Table 7, SEQ ID NOS: 1, 2, 7, 47 and 82 demonstrated at least 25% inhibition of CD40 expression and are therefore preferred compounds of the invention.

EXAMPLE 12: Inhibition of CD40 Expression by Phosphorothioate 2'-MOE Gapmer Oligonucleotides

In accordance with the present invention, a second series of oligonucleotides complementary to mRNA were designed to target different regions of the human CD40 mRNA, using published sequence X60592. The oligonucleotides are shown in Table 8. Target sites are indicated by the beginning or initial nucleotide numbers, as given in the sequence source reference (X60592), to which the oligonucleotide binds.

All compounds in Table 8 are chimeric oligonucleotides ("gapmers") 18 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by four-nucleotide "wings." The wings are composed of 2'-O-(2-methoxyethyl) (2'-MOE) nucleotides. The intersugar (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide. Cytidine residues in the 2'-MOE wings are 5-methylcytidines. Data are averaged from three experiments.

Table 8:

Inhibition of CD40 mRNA Levels by Chimeric Phosphorothioate Oligonucleotides

ISIS#	TARGET SEQUENCE	% Inhibition	SEQ ID
20	19211 18 CCAGGCGGCAGGACCA	75.71	1
	19212 20 GACCAGGCGGCAGGA	77.23	2
	19213 26 AGGTGAGACCAGGCG	80.82	3
	19214 48 CAGAGGCAGACGAAC	23.68	4
	19215 49 GCAGAGGCAGACGAA	45.97	5
25	19216 73 GCAAGCAGCCCCAGAG	65.80	6
	19217 78 GGTCAGCAAGCAGCCC	74.73	7
	19218 84 GACAGCGGTCAGCAAG	67.21	8
	19219 88 GATGGACAGCGGTCAG	65.14	9
	19220 92 TCTGGATGGACAGCGG	78.71	10
30	19221 98 GGTGGTTCTGGATGGA	81.33	11
	19222 101 GTGGGTGGTTCTGGAT	57.79	12
	19223 104 GCAGTGGGTGGTTCTG	73.70	13
	19224 152 CACAAAGAACAGCACT	40.25	14
	19225 156 CTGGCACAAAGAACAG	60.11	15

	19226	162	TCCTGGCTGGCACAAA	10.18	16
	19227	165	CTGTCCTGGCTGGCAC	24.37	17
	19228	176	CTCACCAGTTTCTGTC	22.30	18
	19229	179	TCACTCACCAGTTTCT	40.64	19
5	19230	185	GTGCAGTCACTCACCA	82.04	20
	19231	190	ACTCTGTGCAGTCACT	37.59	21
	19232	196	CAGTGA ACTCTGTGCA	40.26	22
	19233	205	ATTCCGTTTCAGTGAA	56.03	23
	19234	211	GAAGGCATTCCGTTTC	32.21	24
10	19235	222	TTCACCGCAAGGAAGG	61.03	25
	19236	250	CTCTGTTCCAGGTGTCT	62.19	26
	19237	267	CTGGTGGCAGTGTGTC	70.32	27
	19238	286	TGGGGTCGCAGTATTT	0.00	28
	19239	289	GGTTGGGGTCGCAGTA	19.40	29
15	19240	292	CTAGGTTGGGGTCGCA	36.32	30
	19241	318	GGTGCCCTTCTGCTGG	78.91	31
	19242	322	CTGAGGTGCCCTTCTG	69.84	32
	19243	332	GTGTCTGTTTCTGAGG	63.32	33
	19244	334	TGGTGTCTGTTTCTGA	42.83	34
20	19245	345	ACAGGTGCAGATGGTG	73.31	35
	19246	348	TTCACAGGTGCAGATG	47.72	36
	19247	360	GTGCCAGCCTTCTTCA	61.32	37
	19248	364	TACAGTGCCAGCCTTC	46.82	38
	19249	391	GGACACAGCTCTCACA	0.00	39
25	19250	395	TGCAGGACACAGCTCT	52.05	40
	19251	401	GAGCGGTGCAGGACAC	50.15	41
	19252	416	AAGCCGGGCGAGCATG	32.36	42
	19253	432	AATCTGCTTGACCCCA	0.00	43
	19254	446	GAAACCCCTGTAGCAA	0.00	44
30	19255	452	GTATCAGAAACCCCTG	36.13	45
	19256	463	GCTCGCAGATGGTATC	64.65	46
	19257	468	GCAGGGCTCGCAGATG	74.95	47
	19258	471	TGGGCAGGGCTCGCAG	0.00	48
	19259	474	GACTGGGCAGGGCTCG	82.00	49
35	19260	490	CATTGGAGAAGAAGCC	41.31	50
	19261	497	GATGACACATTGGAGA	13.81	51
	19262	500	GCAGATGACACATTGG	78.48	52
	19263	506	TCGAAAGCAGATGACA	59.28	53
	19264	524	GTCCAAGGGTGACATT	70.99	54
40	19265	532	CACAGCTTGTTCCAAGG	0.00	55
	19266	539	TTGGTCTCACAGCTTG	45.92	56
	19267	546	CAGGTCTTTGGTCTCA	63.95	57
	19268	558	CTGTTGCACAACCAGG	82.32	58
	19269	570	GTTTGTGCCTGCCTGTT	70.10	59
45	19270	575	GTCTTGTTTGTGCCTGC	68.95	60
	19271	590	CCACAGACAACATCAG	11.22	61
	19272	597	CTGGGGACCACAGACA	9.04	62
	19273	607	TCAGCCGATCCTGGGG	0.00	63
	19274	621	CACCACCAGGGCTCTC	23.08	64
50	19275	626	GGGATCACCACCAGGG	57.94	65
	19276	657	GAGGATGGCAAACAG	49.14	66

5	19277	668	ACCAGCACCAAGAGG	3.48	67
	19278	679	TTTTGATAAAGACCAG	30.58	68
	19279	703	TATTGGTTGGCTTCTTG	49.26	69
	19280	729	GGGTTCTTGCTTGGGG	13.95	70
	19281	750	GTCGGGAAAATTGATC	54.78	71
10	19282	754	GATCGTCGGGAAAATT	0.00	72
	19283	765	GGAGCCAGGAAGATC	69.47	73
	19284	766	TGGAGCCAGGAAGATC	54.48	74
	19285	780	TGGAGCAGCAGTGTG	15.17	75
	19286	796	GTAAAGTCTCCTGCAC	30.62	76
15	19287	806	TGGCATCCATGTAAAG	65.03	77
	19288	810	CGGTTGGCATCCATGT	34.49	78
	19289	834	CTCTTTGCCATCCTCCT	41.84	79
	19290	861	CTGTCTCTCCTGCACT	25.68	80
	19291	873	GGTGCAGCCTCACTGT	76.27	81
20	19292	910	AACTGCCTGTTTGCCC	63.34	82
	19293	954	CTTCTGCCTGCACCCC	0.00	83
	19294	976	ACTGACTGGGCATAGC	11.55	84

As shown in Table 8, SEQ ID NOS: 1, 2, 3, 6, 7, 8, 9, 10, 11, 12, 13, 15, 20, 23, 25, 26, 27, 31, 32, 33, 35, 37, 40, 41, 46, 47, 49, 52, 53, 54, 57, 58, 59, 60, 65, 71, 73, 74, 77, 81 and 82 demonstrated at least 50% inhibition of CD40 expression and are therefore preferred compounds of the invention.

EXAMPLE 13: Oligonucleotide-Sensitive Sites of the CD40 Target Nucleic Acid

As the data presented in the preceding two Examples shows, several sequences were present in preferred compounds of two distinct oligonucleotide chemistries. Specifically, compounds having SEQ ID NOS: 1, 2, 7, 47 and 82 are preferred in both instances. These compounds map to different regions of the CD40 transcript but nevertheless define accessible sites of the target nucleic acid.

For example, SEQ ID NOS: 1 and 2 overlap each other and both map to the 5'-untranslated region (5'-UTR) of CD40. Accordingly, this region of CD40 is particularly preferred for modulation via sequence-based technologies. Similarly, SEQ ID NOS: 7 and 47 map to the open reading frame of CD40, whereas SEQ ID NO: 82 maps to the 3'-untranslated region (3'-UTR). Thus, the ORF and 3'-UTR of CD40 may be targeted by sequence-based technologies as well.

The reverse complements of the active CD40 compounds are easily determined by those skilled in the art and may be assembled to yield nucleotide sequences corresponding

to accessible sites on the target nucleic acid. For example, the assembled reverse complement of SEQ ID NOS: 1 and 2 is represented below as SEQ ID NO:92:

5'- AGTGGTCCTGCCGCCTGGTC -3' SEQ ID NO:92

TCACCAGGACGGCGGACC -5' SEQ ID NO:1

5 ACCAGGACGGCGGACCAG -5' SEQ ID NO:2

Through multiple iterations of the process of the invention, more extensive “footprints” are generated. A library of this information is compiled and may be used by those skilled in the art in a variety of sequence-based technologies to study the molecular and biological functions of CD40 and to investigate or confirm its role in various diseases and disorders.

EXAMPLE 14: Site Selection Program

In a preferred embodiment of the invention, illustrated in Figure 20, an application is deployed which facilitates the selection process for determining the target positions of the oligos to be synthesized, or “sites.” This program is written using a three-tiered object-oriented approach. All aspects of the software described, therefore, are tightly integrated with the relational database. For this reason, explicit database read and write steps are not shown. It should be assumed that each step described includes database access. The description below illustrates one way the program can be used. The actual interface allows users to skip from process to process at will, in any order.

Before running the site picking program, the target must have all relevant properties computed as described previously and indicated in process step 2204. When the site picking program is launched at process step 2206 the user is presented with a panel showing targets which have previously been selected and had their properties calculated. The user selects one target to work with at process step 2208 and proceeds to decide if any derived properties will be needed at process step 2210. Derived properties are calculated by performing mathematical operations on combinations of pre-calculated properties as defined by the user at process step 2212.

The derived properties are made available as peers with all the pre-calculated properties. The user selects one of the properties to view plotted versus target position at process step 2214. This graph is shown above a linear representation of the target. The

horizontal or position axis of both the graph and target are linked and scalable by the user. The zoom range goes from showing the full target length to showing individual target bases as letters and individual property points. The user next selects a threshold value below or above which all sites will be eliminated from future consideration at process step 2216. The user decides whether to eliminate more sites based on any other properties at process step 2218. If they choose to eliminate more, they return to pick another property to display at process step 2214 and threshold at process step 2216.

After eliminating sites, the user selects from the remaining list by choosing any property at process step 2220 and then choosing a manual or automatic selection technique at process step 2222. In the automatic technique, the user decides whether they want to pick from maxima or minima and the number of maxima or minima to be selected as sites at process step 2224. The software automatically finds and picks the points. When picking manually the user must decide if they wish to use automatic peak finding at process step 2226. If the user selects automatic peak finding, then user must click on the graphed property with the mouse at process step 2236. The nearest maxima or minima, depending on the modifier key held down, to the selected point will be picked as the site. Without the peak finding option, the user must pick a site at process step 2238 by clicking on its position on the linear representation of target.

Each time a site, or group of sites, is picked, a dynamic property is calculated for all possible sites (not yet eliminated) at process step 2230. This property indicates the nearness of the site to a picked site allowing the user to pick sites in subsequent iterations based on target coverage. After new sites are picked, the user determines if the desired number of sites has been picked. If too few sites have been picked the user returns to pick more 2220. If too many sites have been picked, the user may eliminate them by selecting and deleting them on the target display at process step 2234. If the correct number of sites is picked, and the user is satisfied with the set of picked sites, the user registers these sites to the database along with their name, notebook number, and page number at process step 2238. The database time stamps this registration event.

EXAMPLE 15: Site Selection Program

In a preferred embodiment of the invention, illustrated in Figure 21, an application is deployed which facilitates the assignment of specific chemical structure to the complement of the sequence of the sites previously picked and facilitates the registration and ordering of these now fully defined antisense compounds. This program is written using a three-tiered object-oriented approach. All aspects of the software described, therefore, are tightly integrated with the relational database. For this reason, explicit database read and write steps are not shown, it being understood that each step described also includes appropriate database read/write access.

To begin using the oligonucleotide chemistry assignment program, the user launches it at process step **2302**. The user then selects from the previously selected sets of oligonucleotides at process step **2304**, registered to the database in site picker's process step **2238**. Next, the user must decide whether to manually assign the chemistry a base at a time, or run the sites through a template at process step **2306**. If the user chooses to use a template, they must determine if a desired template is available at process step **2308**. If a template is not available with the desired chemistry modifications and the correct length, the user can define one at process step **2314**.

To define a template, the user must select the length of the oligonucleotide the template is to define. This oligonucleotide is then represented as a bar with selectable regions. The user sets the number of regions on the oligonucleotide, and the positions and lengths of these regions by dragging them back and forth on the bar. Each region is represented by a different color.

For each region, the user defines the chemistry modifications for the sugars, the linkers, and the heterocycles at each base position in the region. At least four heterocycle chemistries must be given, one for each of the four possible base types (A, G, C or T or U) in the site sequence the template will be applied to. A user interface is provided to select these chemistries which show the molecular structure of each component selected and its modification name. By pushing on a pop-up list next to each of the pictures, the user may choose from a list of structures and names, those possible to put in this place. For example, the heterocycle that represents the base type G is shown as a two dimensional structure diagram. If the user clicks on the pop-up list, a row of other possible structures

and names is shown. The user drags the mouse to the desired chemistry and releases the mouse. Now the newly selected molecule is displayed as the choice for G type heterocycle modifications.

Once the user has created a template, or selected an existing one, the software
5 applies the template at process step **2312** to each of the complements of the sites in the list. When the templates are applied, it is possible that chemistries will be defined which are impossible to make with the chemical precursors presently used on the automatic synthesizer. To check this, a database is maintained of all precursors previously designed, and their availability for automated synthesis. When the templates are applied, the
10 resulting molecules are tested at process step **2316** against this database to see if they are readily synthesized.

If a molecule is not readily synthesized, it is added to a list that the user inspects. At process step **2318**, the user decides whether to modify the chemistry to make it compatible with the currently recognized list of available chemistries or to ignore it. To
15 modify a chemistry, the user must use the base at a time interface at process step **2322**. The user can also choose to go directly to this step, bypassing templates all together at process step **2306**.

The base at a time interface at process step **2322** is very similar to the template editor at process step **2314** except that instead of specifying chemistries for regions, they
20 are defined one base at a time. This interface also differs in that it dynamically checks to see if the design is readily synthesized as the user makes selections. In other words, each choice made limits the choices the software makes available on the pop-up selection lists. To accommodate this function, an additional choice is made available on each pop-up of "not defined." For example, this allows the user to inhibit linker choice from restricting
25 the sugar choices by first setting the linker to "not defined." The user would then pick the sugar, and then pick from the remaining linker choices available.

Once all of the sites on the list are assigned chemistries or dropped, they are registered at process step **2324** to a commercial chemical structure database. Registering to this database makes sure the structure is unique, assigns it a new identifier if it is
30 unique, and allows future structure and substructure searching by creating various hash-tables. The compound definition is also stored at process step **2326** to various hash tables

referred to as chemistry/position tables. These allow antisense compound searching and categorization based on oligonucleotide chemistry modification sequences and equivalent base sequences.

The results of the registration are displayed at process step **2328** with the new IDs
 5 if they are new compounds and with the old IDs if they have been previously registered. The user next selects which of the compounds processed they wish to order for synthesis at process step **2330** and registers an order list at process step **2332** by including scientist name, notebook number and page number. The database time-stamps this entry. The user may then choose at process step **2334**, to quit the program at process step **2338**, go back to
 10 the beginning and choose a new site list to work with process step **2304**, or start the oligonucleotide ordering interface at process step **2336**.

EXAMPLE 16: Gene Walk to Optimize Oligonucleotide Sequence

A gene walk is executed using a CD40 antisense oligonucleotide having
 15 SEQ ID NO:15 (5'-CTGGCACAAAGAACAGCA-3'). In effecting this gene walk, the following parameters are used:

Gene Walk Parameter	Entered value
Oligonucleotide Sequence ID:	15
Name of Gene Target:	CD40
20 Scope of Gene Walk:	20
Sequence Shift Increment:	1

Entering these values and effecting the gene walk centered on SEQ ID NO: 15
 automatically generates the following new oligonucleotides:

Table 9:

Oligonucleotide Generated By Gene Walk

SEQ ID	Sequence
93	GAACAGCACTGACTG
94	AGAACAGCACTGACT
95	AAGAACAGCACTGAC
96	AAAGAACAGCACTGA
97	CAAAGAACAGCACTG

	98	ACAAAGAACAGCACT
	14	CACAAAGAACAGCAC
	100	GCACAAAGAACAGCA
	101	GGCACAAAGAACAGC
5	102	TGGCACAAAGAACAG
	15	CTGGCACAAAGAACA
	103	GCTGGCACAAAGAAC
	104	GGCTGGCACAAAGAA
	105	TGGCTGGCACAAAGA
10	106	CTGGCTGGCACAAAG
	107	CCTGGCTGGCACAAA
	16	TCCTGGCTGGCACAA
	109	GTCCTGGCTGGCACAA
	110	TGTCCTGGCTGGCACAA
15	17	CTGTCCTGGCTGGCAC
	112	TCTGTCCTGGCTGGCAA

The list shown above contains 20 oligonucleotide sequences directed against the CD40 nucleic acid sequence. They are ordered by the position along the CD40 sequence at which the 5' terminus of each oligonucleotide hybridizes. Thus, the first ten oligonucleotides are single-base frame shift sequences directed against the CD40 sequence upstream of compound SEQ ID NO: 15 and the latter ten are single-base frame shift sequences directed against the CD40 sequence downstream of compound SEQ ID NO: 15.

EXAMPLE 17: Automated Assay of RhoC Oligonucleotide Activity

RhoC, a member of the Rho subfamily of small GTPases, is a protein that has been shown to be involved in a diverse set of signaling pathways including the ultimate regulation of the dynamic organization of the cytoskeleton.

Oligonucleotides were designed as described in Example 2, synthesized as described in Examples 3 through 8, analyzed as described in Examples 9 and assayed as described in Example 10 except for target specific primer and probes. RhoC probes and primers were designed to hybridize to the human RhoC sequence, using published sequence information (GenBank accession number L25081, incorporated herein by reference as SEQ ID NO:113).

For RhoC the PCR primers were:
forward primer: TGATGTCATCCTCATGTGCTTCT (SEQ ID NO: 114)

reverse primer: CCAGGATGATGGGCACGTT (SEQ ID NO: 115) and the PCR probe was: FAM-CGACAGCCCTGACAGCCTGGAAA-TAMRA (SEQ ID NO: 116) where FAM (PE-Applied Biosystems, Foster City, CA) is the fluorescent reporter dye) and TAMRA (PE-Applied Biosystems, Foster City, CA) is the quencher dye.

5

EXAMPLE 18: Antisense inhibition of RhoC expression- phosphorothioate oligodeoxynucleotides

In accordance with the present invention, a series of oligonucleotides were designed to target different regions of the human RhoC RNA, using published sequences (GenBank accession number L25081, incorporated herein by reference as SEQ ID NO: 113). The oligonucleotides are shown in Table 10. Target sites are indicated by nucleotide numbers, as given in the sequence source reference (Genbank accession no. L25081), to which the oligonucleotide binds. All compounds in Table 10 are oligodeoxynucleotides with phosphorothioate backbones (internucleoside linkages) throughout. The compounds were analyzed for effect on RhoC mRNA levels by quantitative real-time PCR as described in other examples herein. Data are averages from three experiments. If present, "N.D." indicates "no data".

Table 10

Inhibition of RhoC mRNA levels by phosphorothioate oligodeoxynucleotides

ISIS#	REGION	TARGET SITE	SEQUENCE	% Inhibition	SEQ ID NO.
25304	5' UTR	4	gagctgagatgaagtcaa	29	117
25305	5' UTR	44	gctgaagttcccaggctg	25	118
25306	5' UTR	47	ccggtgaagtcccagg	42	119
25307	Coding	104	ggcaccatcccaacgat	81	120
25308	Coding	105	aggcaccatcccaacga	81	121
25309	Coding	111	tccacaggcaccatccc	70	122
25310	Coding	117	aggtcttccacaggcac	40	123
25311	Coding	127	atgaggaggcaggcttc	41	124
25312	Coding	139	ttgctgaagacgatgagg	23	125
25313	Coding	178	tcaaagacagtagggacg	0	126
25314	Coding	181	ttctcaaagacagtaggg	2	127
25315	Coding	183	agttctcaaagacagtag	38	128
25316	Coding	342	tgtttccaggctgtcag	59	129

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	25317	Coding	433	tcgtcttgcctcaggtcc	79	130
	25318	Coding	439	gtgtgctcgtcttgctc	67	131
	25319	Coding	445	ctcctggtgtgctcgtct	67	132
	25320	Coding	483	cagaccgaacgggctcct	65	133
5	25321	Coding	488	ttcctcagaccgaacggg	57	134
	25322	Coding	534	actcaaggtagccaaagg	33	135
	25323	Coding	566	ctccgcactccctcctt	91	136
	25324	Coding	575	ctcaaacacctcccgac	34	137
	25325	Coding	581	ggccatctcaaacacctc	64	138
10	25326	Coding	614	cttgcttgaggacctg	72	139
	25327	Coding	625	cccctccgacgcttgct	66	140
	25328	3' UTR	737	gtatggagccctcaggag	60	141
	25329	3' UTR	746	gagccttcagtatggagc	63	142
	25330	3' UTR	753	gaaaatggagccttcagt	24	143
15	25331	3' UTR	759	ggaactgaaaatggagcc	2	144
	25332	3' UTR	763	ggaggggaactgaaaatgg	13	145
	25333	3' UTR	766	gcaggagggaactgaaaa	27	146
	25334	3' UTR	851	agggcagggcataggcgt	31	147
	25335	3' UTR	854	ggaagggcagggcatagg	21	148
20	25336	3' UTR	859	catgaggaagggcagggc	0	149
	25337	3' UTR	920	taaagtgtgtgtgtga	39	150
	25338	3' UTR	939	cctgtgagccagaagtgt	69	151
	25339	3' UTR	941	ttcctgtgagccagaagt	69	152
	25340	3' UTR	945	cactttcctgtgagccag	82	153
25	25341	3' UTR	948	agacacttcctgtgagc	69	154
	25342	3' UTR	966	actctgggtccctactgc	20	155
	25343	3' UTR	992	tgcagaaacaactccagg	0	156

Example 19: Antisense inhibition of RhoC expression- phosphorothioate 2'-MOE gapmer oligonucleotides

In accordance with the present invention, a second series of oligonucleotides targeted to human RhoC were synthesized. The oligonucleotide sequences are shown in Table 11. Target sites are indicated by nucleotide numbers, as given in the sequence source reference (Genbank accession No. L25081), to which the oligonucleotide binds.

All compounds in Table 11 are chimeric oligonucleotides ("gapmers") 18 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by four-nucleotide "wings". The wings are composed of 2'-methoxyethyl (2'-MOE) nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the

oligonucleotide. Cytidine residues in the 2'-MOE wings are 5-methylcytidines.

Data were obtained by real-time quantitative PCR as described in other examples herein and are averaged from three experiments. If present, "N.D." indicates "no data".

5

Table 11

**Inhibition of RhoC mRNA levels by chimeric phosphorothioate oligonucleotides
having 2'-MOE wings and a deoxy gap**

	ISIS#	REGION	TARGET SITE	SEQUENCE	% Inhibition	SEQ ID NO.
10	25344	5' UTR	4	gagctgagatgaagtcaa	0	117
	25345	5' UTR	44	gctgaagttcccaggtcg	35	118
	25346	5' UTR	47	ccggctgaagttcccagg	53	119
	25347	Coding	104	ggcaccatccccaacgat	50	120
	25348	Coding	105	aggcaccatccccaacga	56	121
15	25349	Coding	111	tcccacaggcaccatccc	4	122
	25350	Coding	117	aggtcttcccacaggcac	11	123
	25351	Coding	127	atgaggaggcagggtctc	6	124
	25352	Coding	139	ttgctgaagacgatgagg	15	125
	25353	Coding	178	tcaaagacagtagggacg	32	126
20	25354	Coding	181	ttctcaaagacagtaggg	7	127
	25355	Coding	183	agttctcaaagacagtag	39	128
	25356	Coding	342	tgttttccaggctgtcag	59	129
	25357	Coding	433	tcgtcttgccctcaggctc	48	130
	25358	Coding	439	gtgtgctcgtcttgccctc	36	131
25	25359	Coding	445	ctcctgggtgtgctcgtct	61	132
	25360	Coding	483	cagaccgaacgggctcct	50	133
	25361	Coding	488	ttctcagaccgaacggg	14	134
	25362	Coding	534	actcaaggtagccaaagg	32	135
	25363	Coding	566	ctcccgcactccctcctt	21	136
30	25364	Coding	575	ctcaaacacctcccgcac	9	137
	25365	Coding	581	ggccatctcaaacacctc	66	138
	25366	Coding	614	cttggtcttgccggacctg	61	139
	25367	Coding	625	cccctccgacgctgttc	0	140
	25368	3' UTR	737	gtatggagccctcaggag	28	141
35	25369	3' UTR	746	gagccttcagtatggagc	32	142
	25370	3' UTR	753	gaaatggagccttcagt	0	143
	25371	3' UTR	759	ggaactgaaatggagcc	40	144
	25372	3' UTR	763	ggagggaactgaaaatgg	45	145
	25373	3' UTR	766	gcaggagggaactgaaaa	35	146
40	25374	3' UTR	851	agggcaggcataggcgt	5	147
	25375	3' UTR	854	ggaagggcaggcatagg	0	148

5	25376	3' UTR	859	catgaggaagggcagggc	0	149
	25377	3' UTR	920	taaagtgctggtgtga	20	150
	25378	3' UTR	939	cctgtgagccagaagtgt	67	151
	25379	3' UTR	941	ttcctgtgagccagaagt	61	152
	25380	3' UTR	945	cactttcctgtgagccag	80	153
	25381	3' UTR	948	agacactttcctgtgagc	0	154
	25382	3' UTR	966	actctgggtccctactgc	0	155
	25383	3' UTR	992	tcgagaaacaactccagg	0	156

10 **EXAMPLE 20: Automated Assay of Cellular Inhibitor of Apoptosis-2 Expression Oligonucleotide Activity**

Cellular Inhibitor of Apoptosis-2 (also known as c-IAP-2, apoptosis inhibitor 2, API-2, hIAP-1, and MIHC) is a member of the inhibitor of apoptosis (IAP) family of anti-apoptotic proteins which interfere with the transmission of intracellular death signals.

15 Oligonucleotides were designed as described in Example 2, synthesized as described in Examples 3 through 8, analyzed as described in Examples 9 and assayed as described in Example 10 except for target specific primer and probes. Cellular Inhibitor of Apoptosis-2 probes and primers were designed to hybridize to the human Cellular Inhibitor of Apoptosis-2 sequence, using published sequence information (GenBank accession number U37546, incorporated herein by reference as SEQ ID NO:157).

20 For Cellular Inhibitor of Apoptosis-2 the PCR primers were:
forward primer: GGAAGTCAAGGTGTTGGGAATCTG (SEQ ID NO: 158)
reverse primer: CAAGTACTCACACCTTGGAAACCA (SEQ ID NO: 159) and the PCR probe was: FAM-AGATGATCCATGGGTTCAACATGCCAA-TAMRA (SEQ ID NO: 25 160) where FAM (PE-Applied Biosystems, Foster City, CA) is the fluorescent reporter dye) and TAMRA (PE-Applied Biosystems, Foster City, CA) is the quencher dye.

EXAMPLE 21: Antisense inhibition of Cellular Inhibitor of Apoptosis-2 expression-phosphorothioate oligodeoxynucleotides

30 In accordance with the present invention, a series of oligonucleotides were designed to target different regions of the human Cellular Inhibitor of Apoptosis-2 RNA, using published sequences (GenBank accession number U37546, incorporated herein by reference as SEQ ID NO: 157). The oligonucleotides are shown in Table 12. Target sites

are indicated by nucleotide numbers, as given in the sequence source reference (Genbank accession no. U37546), to which the oligonucleotide binds. All compounds in Table 12 are oligodeoxynucleotides with phosphorothioate backbones (internucleoside linkages) throughout. The compounds were analyzed for effect on Cellular Inhibitor of Apoptosis-2 mRNA levels by quantitative real-time PCR as described in other examples herein. Data are averages from three experiments. If present, "N.D." indicates "no data".

Table 12

Inhibition of Cellular Inhibitor of Apoptosis-2 mRNA levels by phosphorothioate oligodeoxynucleotides

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ISIS#	REGION	TARGET SITE	SEQUENCE	% Inhibition	SEQ ID NO.
23412	5' UTR	3	actgaagacatttgaat	62	161
23413	5' UTR	37	cttagaggtagctaaaat	29	162
23414	5' UTR	49	gcacttttatttcttaga	70	163
23415	5' UTR	62	attttaattagaagcact	0	164
23416	5' UTR	139	accatatttcactgattc	70	165
23417	5' UTR	167	ctaactcaaaggaggaaa	0	166
23418	5' UTR	175	cacaagacctaactcaaa	27	167
23419	5' UTR	268	gctctgctgtcaagtgtt	57	168
23420	5' UTR	303	tgtgtgactcatgaagct	23	169
23421	5' UTR	335	ttcagtggcattcaatca	23	170
23422	5' UTR	357	cttctccaggctactaga	50	171
23423	5' UTR	363	ggccaactctccagget	65	172
23424	5' UTR	437	taaaacccttcacagaag	0	173
23425	5' UTR	525	ttaaggaagaatacaca	0	174
23426	5' UTR	651	gcattggcttgcctttat	0	175
23427	Coding	768	caaacgtgttgccgcttt	35	176
23428	Coding	830	agcaggaaaagtgaata	0	177
23429	Coding	1015	ttaacggaatttagactc	0	178
23430	Coding	1064	atttggtactgaagaagg	0	179
23431	Coding	1118	agagccacggaaatatcc	9	180
23432	Coding	1168	aaatcttgatttgcctg	7	181
23433	Coding	1231	gtaagtaactctggcattt	0	182
23434	Coding	1323	agcaagccactctgtctc	50	183
23435	Coding	1436	tgaagtgtcttgaagctg	0	184
23436	Coding	1580	tttgacatcatcactgtt	0	185
23437	Coding	1716	tggttggaacttgacgga	0	186
23438	Coding	1771	tcattctcctgggctgtct	40	187

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	23439	Coding	1861	gcagcattaatcacagga	0	188
	23440	Coding	2007	tttctctctctcttccc	10	189
	23441	Coding	2150	aacatcatgttctgttc	9	190
	23442	Coding	2273	atataacacagcttcagc	0	191
5	23443	Coding	2350	aattgttcttccactgt	0	192
	23444	Coding	2460	aagaaggagcacatctt	70	193
	23445	3' UTR	2604	gaaaccaaattaggataa	12	194
	23446	3' UTR	2753	tgtagtgctacctcttt	69	195
	23447	3' UTR	2779	ctgaaatttgattgaat	14	196
10	23448	3' UTR	2795	tacaatttcaataatget	38	197
	23449	3' UTR	2920	gggtctcagtatgctgcc	21	198
	23450	3' UTR	3005	ccttcgatgtataggaca	0	199
	23451	3' UTR	3040	catgtccctaaaatgtca	0	200

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EXAMPLE 22: Antisense inhibition of Cellular Inhibitor of Apoptosis-2 expression-phosphorothioate 2'-MOE gapmer oligonucleotides

In accordance with the present invention, a second series of oligonucleotides targeted to human Cellular Inhibitor of Apoptosis-2 were synthesized. The oligonucleotide sequences are shown in Table 13. Target sites are indicated by nucleotide numbers, as given in the sequence source reference (Genbank accession no. U37546), to which the oligonucleotide binds.

All compounds in Table 13 are chimeric oligonucleotides ("gapmers") 18 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by four-nucleotide "wings". The wings are composed of 2'-methoxyethyl (2'-MOE)nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide. Cytidine residues in the 2'-MOE wings are 5-methylcytidines.

Data were obtained by real-time quantitative PCR as described in other examples herein and are averaged from three experiments. If present, "N.D." indicates "no data".

35

Table 13

Inhibition of Cellular Inhibitor of Apoptosis-2 mRNA levels by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap

5	ISIS#	REGION	TARGET SITE	SEQUENCE	% Inhibition	SEQ ID NO.
	23452	5' UTR	3	actgaagacattttgaat	35	161
	23453	5' UTR	37	cttagaggtagctaaaat	26	162
	23454	5' UTR	49	gcacttttatttcttaga	76	163
10	23455	5' UTR	62	attttaattagaagcact	0	164
	23456	5' UTR	139	accatatttctactgattc	0	165
	23457	5' UTR	167	ctaactcaaaggaggaaa	5	166
	23458	5' UTR	175	cacaagacctactcaaa	0	167
	23459	5' UTR	268	gctctgctgtcaagtgtt	57	168
15	23460	5' UTR	303	tgtgtgactcatgaagct	67	169
	23461	5' UTR	335	ttcagtggcattcaatca	59	170
	23462	5' UTR	357	cttctccaggctactaga	0	171
	23463	5' UTR	363	ggtaaccttctccaggct	75	172
	23464	5' UTR	437	taaaacccttcacagaag	11	173
20	23465	5' UTR	525	ttaaggaagaaatacaca	0	174
	23466	5' UTR	651	gcatggctttgctttat	46	175
	23467	Coding	768	caaacgtgttggcgctt	47	176
	23468	Coding	830	agcaggaaaagtgggaata	39	177
	23469	Coding	1015	ttaacggaatttagactc	12	178
25	23470	Coding	1064	atttggtactgaagaagg	34	179
	23471	Coding	1118	agagccacggaaatatcc	54	180
	23472	Coding	1168	aaatcttgatttgctctg	34	181
	23473	Coding	1231	gtaagtaactctggcattt	0	182
	23474	Coding	1323	agcaagccactctgtctc	42	183
30	23475	Coding	1436	tgaagtgtcttgaagctg	0	184
	23476	Coding	1580	tttgacatcatcactgtt	57	185
	23477	Coding	1716	tggcttgaacttgacgga	23	186
	23478	Coding	1771	tcattctctgggctgtct	66	187
	23479	Coding	1861	gcagcattaatcacagga	65	188
35	23480	Coding	2007	tttctctctctctctccc	0	189
	23481	Coding	2150	aacatcatgttcttggtc	13	190
	23482	Coding	2273	atataacacagcttcagc	0	191
	23483	Coding	2350	aattgttcttccactggt	60	192
	23484	Coding	2460	aagaaggagcacaaatctt	65	193
40	23485	3' UTR	2604	gaaaccaaattaggataa	0	194
	23486	3' UTR	2753	tgtagtgtctacctctttt	73	195
	23487	3' UTR	2779	ctgaaattttgattgaat	4	196

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23488	3' UTR	2795	tacaatttcaataatgct	0	197
23489	3' UTR	2920	gggtctcagtatgctgcc	42	198
23490	3' UTR	3005	ccttcgatgtataggaca	71	199
23491	3' UTR	3040	catgtccctaaaatgtca	45	200

5

EXAMPLE 23: Automated Assay of ELK-1 Oligonucleotide Activity

ELK-1 (also known as p62TCF) is a member of the ternary complex factor (TCF) subfamily of Ets domain proteins and utilizes a bipartite recognition mechanism mediated by both protein-DNA and protein-protein interactions. This results in gene regulation not only by direct DNA binding but also by indirect DNA binding through recruitment by other factors (Rao et al., *Science*, **1989**, 244, 66-70). The formation of ternary complexes with an array of proteins allows the differential regulation of many genes. The mechanism by which ELK-1 controls various signal transduction pathways involves regulating the activity of the Egr-1, pip92, nur77 and c-fos promoters by binding to the serum response element (SRE) in these promoters in response to extracellular stimuli such as growth factors, mitogens and oncogene products (Sharrocks et al., *Int. J. Biochem. Cell Biol.*, **1997**, 29, 1371-1387). ELK-1 has also been shown to mediate other functions within the cell including apoptosis.

Oligonucleotides were designed as described in Example 2, synthesized as described in Examples 3 through 8, analyzed as described in Examples 9 and assayed as described in Example 10 except for target specific primer and probes. ELK-1 probes and primers were designed to hybridize to the human ELK-1 sequence, using published sequence information (GenBank accession number M25269, incorporated herein by reference as SEQ ID NO:201).

For ELK-1 the PCR primers were:

forward primer: GCAAGGCAATGGCCACAT (SEQ ID NO: 202)

reverse primer: CTCCTCTGCATCCACCAGCTT (SEQ ID NO: 203) and the PCR probe was: FAM-TCTCCTGGACTTCACGGGATGGTGGT-TAMRA (SEQ ID NO: 204)

where FAM (PE-Applied Biosystems, Foster City, CA) is the fluorescent reporter dye) and TAMRA (PE-Applied Biosystems, Foster City, CA) is the quencher dye.

30

EXAMPLE 24: Antisense inhibition of ELK-1 expression-phosphorothioate oligodeoxynucleotides

In accordance with the present invention, a series of oligonucleotides were designed to target different regions of the human ELK-1 RNA, using published sequences (GenBank accession number M25269, incorporated herein by reference as SEQ ID NO: 201). The oligonucleotides are shown in Table 14. Target sites are indicated by nucleotide numbers, as given in the sequence source reference (Genbank accession no. M25269), to which the oligonucleotide binds. All compounds in Table 14 are oligodeoxynucleotides with phosphorothioate backbones (internucleoside linkages) throughout. The compounds were analyzed for effect on ELK-1 mRNA levels by quantitative real-time PCR as described in other examples herein. Data are averages from three experiments. If present, "N.D." indicates "no data".

Table 14

Inhibition of ELK-1 mRNA levels by phosphorothioate oligodeoxynucleotides

ISIS#	REGION	TARGET SITE	SEQUENCE	% Inhibition	SEQ ID NO.
20	24752	5' UTR 11	cccctgcgtttccctaca	15	205
	24753	5' UTR 50	ggtggtggtggcgggtggc	29	206
	24754	5' UTR 139	ggcgttggcaatgttggc	82	207
	24755	5' UTR 167	aagttgaggctgtgtgta	0	208
	24756	5' UTR 189	aggccacggacgggtctc	92	209
	24757	5' UTR 229	gattgattcgctacgatg	71	210
25	24758	5' UTR 255	gggatgcggaggagtgcg	74	211
	24759	5' UTR 289	agtgtcacgccatcca	22	212
	24760	Coding 328	aaactgccacagcgtcac	64	213
	24761	Coding 381	gaagtccaggagatgatg	62	214
	24762	Coding 395	caccaccatcccgtgaag	88	215
30	24763	Coding 455	tcttgttcttgcgtagtc	62	216
	24764	Coding 512	tgttcttgcatagtagt	52	217
	24765	Coding 527	tcaccttgcggatgatgt	57	218
	24766	Coding 582	gagcaccctgcgacctca	72	219
	24767	Coding 600	ggcgggcagtcctcagtg	82	220
35	24768	Coding 787	ggtgaagggtggaatagag	58	221
	24769	Coding 993	tccgatttcaggttggg	55	222
	24770	Coding 1110	ttggtggttctgcgaca	67	223

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5	24771	Coding	1132	tggagggacttctggctc	69	224
	24772	Coding	1376	gcgtaggaagcagggatg	34	225
	24773	Coding	1440	gtgctccagaagtgaatg	64	226
	24774	Coding	1498	actggatggaaactggaa	34	227
	24775	Coding	1541	ggccatccacgtgatag	74	228
10	24776	3' UTR	1701	ccaccacaatcagagcat	74	229
	24777	3' UTR	1711	gatccccacccaccaca	16	230
	24778	3' UTR	1765	tgtttctgtggaggaga	48	231
	24779	3' UTR	1790	aaacagagaagtgtgga	11	232
	24780	3' UTR	1802	gggactgacagaaaacag	0	233
15	24781	3' UTR	1860	ataaataataaaccgcc	18	234
	24782	3' UTR	1894	gttaggtcaggctcatcc	56	235
	24783	3' UTR	1974	gttctcaagccagacctc	52	236
	24784	3' UTR	1992	aataaagaaagaaaggtc	41	237
	24785	3' UTR	2006	agggcaggctgagaaata	29	238
20	24786	3' UTR	2053	cttctactcacatccaaa	54	239
	24787	3' UTR	2068	caaaacaaactaactctt	24	240
	24788	3' UTR	2080	ggaataataaaaacaaac	40	241
	24789	3' UTR	2107	ttctcctggaccctga	93	242
	24790	3' UTR	2161	ccaagggtgtgattcttc	81	243
	24791	3' UTR	2200	tgtctgagagaaaggttg	55	244

EXAMPLE 25: Antisense inhibition of ELK-1 expression- phosphorothioate 2'-MOE gapmer oligonucleotides

25 In accordance with the present invention, a second series of oligonucleotides targeted to human ELK-1 were synthesized. The oligonucleotide sequences are shown in Table 15. Target sites are indicated by nucleotide numbers, as given in the sequence source reference (Genbank accession no. M25269), to which the oligonucleotide binds.

30 All compounds in Table 15 are chimeric oligonucleotides ("gapmers") 18 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by four-nucleotide "wings". The wings are composed of 2'-methoxyethyl (2'-MOE)nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide. Cytidine residues in the 2'-MOE wings are 5-methylcytidines.

35 Data were obtained by real-time quantitative PCR as described in other examples herein and are averaged from three experiments. If present, "N.D." indicates "no data".

Table 15

**Inhibition of ELK-1 mRNA levels by chimeric phosphorothioate oligonucleotides
having 2'-MOE wings and a deoxy gap**

	ISIS#	REGION	TARGET SITE	SEQUENCE	% Inhibition	SEQ ID NO.
5	24792	5' UTR	11	cccctgcgtttccctaca	23	205
	24793	5' UTR	50	ggtggtggtggcggtggc	80	206
	24794	5' UTR	139	ggcgttggcgaatgttggc	91	207
	24795	5' UTR	167	aagttgaggctgtgtgta	27	208
10	24796	5' UTR	189	aggccacggacgggtctc	79	209
	24797	5' UTR	229	gattgattcgctacgatg	69	210
	24798	5' UTR	255	gggatgcggaggagtgcg	42	211
	24799	5' UTR	289	agtgtcacgccatccca	45	212
	24800	Coding	328	aaactgccacagcgtcac	57	213
15	24801	Coding	381	gaagtcacgagatgatg	55	214
	24802	Coding	395	caccaccatcccgtgaag	41	215
	24803	Coding	455	tcttgttcttgcgtagtc	80	216
	24804	Coding	512	tgttctgtcatagtagt	65	217
	24805	Coding	527	tcaccttgcggatgatgt	70	218
20	24806	Coding	582	gagcaccctgcgacctca	64	219
	24807	Coding	600	ggcggggcagtcctcagt	67	220
	24808	Coding	787	ggtgaaggtggaatagag	45	221
	24809	Coding	993	tccgatttcagggttgagg	75	222
	24810	Coding	1110	ttggtggtttctggcaca	82	223
25	24811	Coding	1132	tggaggggacttctggctc	60	224
	24812	Coding	1376	gcgttaggaagcagggatg	49	225
	24813	Coding	1440	gtgctccagaagtgaatg	71	226
	24814	Coding	1498	actggatggaaactggaa	62	227
	24815	Coding	1541	ggccatccacgtgatag	78	228
30	24816	3' UTR	1701	ccaccacaatcagagcat	54	229
	24817	3' UTR	1711	gatccccaccccaccaca	44	230
	24818	3' UTR	1765	tgttttctgtggaggaga	74	231
	24819	3' UTR	1790	aaacagagaagttgtgga	64	232
	24820	3' UTR	1802	gggactgacagaaaacag	16	233
35	24821	3' UTR	1860	ataaataaataaaccgcc	38	234
	24822	3' UTR	1894	gttaggtcaggctcatcc	59	235
	24823	3' UTR	1974	gttctcaagccagacctc	62	236
	24824	3' UTR	1992	aataaagaaagaaaggtc	35	237
	24825	3' UTR	2006	agggcaggctgagaaata	0	238
40	24826	3' UTR	2053	cttctactcacatccaaa	46	239
	24827	3' UTR	2068	caaaacaaactaactctt	38	240
	24828	3' UTR	2080	ggaataataaaaacaaaac	37	241

24829	3' UTR	2107	ttcttcttgaccctga	71	242
24830	3' UTR	2161	ccaagggtgtgattcttc	88	243
24831	3' UTR	2200	tgcttgagagaaagggtg	65	244

5

EXAMPLE 26: Automated Assay of Gi alpha proteins Oligonucleotide Activity

G-alpha-11 is a member of the Gq subfamily of G proteins whose primary function is to activate PLC-b isoforms producing second messengers and affecting intracellular calcium stores.

10

Oligonucleotides were designed as described in Example 2, synthesized as described in Examples 3 through 8, analyzed as described in Examples 9 and assayed as described in Example 10 except for target specific primer and probes. G-alpha-11 probes and primers were designed to hybridize to the human G-alpha-11 sequence, using published sequence information (GenBank accession number AF011497, incorporated herein by reference as SEQ ID NO:245). For G-alpha-11 the PCR primers were: forward primer: TGACCACCTTCGAGCATCAG (SEQ ID NO: 246) reverse primer: CGGTCGTAGCATTCCTGGAT (SEQ ID NO: 247) and the PCR probe was: FAM-TCAGTGCCATCAAGACCCTGTGGGAG-TAMRA (SEQ ID NO: 248) where FAM (PE-Applied Biosystems, Foster City, CA) is the fluorescent reporter dye) and TAMRA (PE-Applied Biosystems, Foster City, CA) is the quencher dye.

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EXAMPLE 27: Antisense inhibition of G-alpha-11 expression- phosphorothioate oligodeoxynucleotides

In accordance with the present invention, a series of oligonucleotides were designed to target different regions of the human G-alpha-11 RNA, using published sequences (GenBank accession number AF011497, incorporated herein by reference as SEQ ID NO: 245). The oligonucleotides are shown in Table 16. Target sites are indicated by nucleotide numbers, as given in the sequence source reference (Genbank accession no. AF011497), to which the oligonucleotide binds. All compounds in Table 16 are oligodeoxynucleotides with phosphorothioate backbones (internucleoside linkages) throughout. The compounds were analyzed for effect on G-alpha-11 mRNA levels by quantitative real-time PCR as described in other examples herein. Data are averages from

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three experiments. If present, "N.D." indicates "no data".

Table 16

Inhibition of G-alpha-11 mRNA levels by phosphorothioate oligodeoxynucleotides

	ISIS#	REGION	TARGET SITE	SEQUENCE	% Inhibition	SEQ ID NO.
5	20576	Coding	1	gatggactccagagtcac	0	249
	20577	Coding	6	gccatgatggactccaga	75	250
	20578	Coding	9	cacgccatgatggactcc	0	251
	20579	Coding	25	ctcatcgctcaggcaaca	61	252
10	20580	Coding	31	cttcacctcatcgctcag	20	253
	20581	Coding	36	gactccttcacctcatcg	15	254
	20582	Coding	45	atccgcttgactccttc	17	255
	20583	Coding	50	cgttgatccgcttgact	0	256
	20584	Coding	61	ctcgatctcggcggtgat	0	257
15	20585	Coding	77	cccgccgagctgcttct	58	258
	20586	Coding	106	cttgagctcgcgccgggc	31	259
	20587	Coding	116	gcagcagcagcttgagct	0	260
	20588	Coding	127	gcccgtgccgagcagcag	0	261
	20589	Coding	146	acgtgctctcccgtct	28	262
20	20590	Coding	159	atctgcttgatgaacgtg	0	263
	20591	Coding	162	cgcactgcttgatgaac	0	264
	20592	Coding	184	gtagccggcgccgtggat	1	265
	20593	Coding	197	tgtcctcctccgagtagc	0	266
	20594	Coding	199	cttgctcctccgagta	79	267
25	20595	Coding	207	aagccgcgcttgctctcc	56	268
	20596	Coding	222	tagacgagcttggtgaag	0	269
	20597	Coding	230	tgttctggtagacgagct	0	270
	20598	Coding	242	tggcggtgaagatgttct	0	271
	20599	Coding	258	cggatcatggcctgcatg	1	272
30	20600	Coding	271	cgtctccatggcccgat	49	273
	20601	Coding	285	tagaggatcttgagcgtc	0	274
	20602	Coding	287	tgtagaggatcttgagcg	0	275
	20603	Coding	297	tgctcgacttgtagagg	7	276
	20604	Coding	306	gccttgcttgctgtac	25	277
35	20605	Coding	309	ttggccttgctgctcg	0	278
	20606	Coding	319	caggagcgattggcctt	0	279
	20607	Coding	340	ctccacgtccacctccg	69	280
	20608	Coding	349	ggcaccttctccacgtc	27	281
	20609	Coding	362	gatgctcgaagggtgtca	33	282
40	20610	Coding	373	actgacgtactgatgctc	36	283
	20611	Coding	382	cttgatggcactgacgta	78	284
	20612	Coding	388	cagggtcttgatggcact	0	285

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5	20613	Coding	409	ctggatgccccgggtcctc	0	286
	20614	Coding	411	tcctggatgcccggtcc	30	287
	20615	Coding	429	cgctcgcggtcgtagcat	0	288
	20616	Coding	440	gctggtactcgcgctgc	41	289
	20617	Coding	459	tacttggcagagtcggag	34	290
10	20618	Coding	468	gtcaggtagtacttggca	76	291
	20619	Coding	479	ggtcaacgtcggtcaggt	18	292
	20620	Coding	489	gtggcgatgcggtcaacg	1	293
	20621	Coding	503	gcaggtagcccaaggtgg	20	294
	20622	Coding	518	cgtcctgctgggtgggca	40	295
15	20623	Coding	544	ggtggtgggcacgcggac	0	296
	20624	Coding	555	tcgatgatgccggtggtg	0	297
	20625	Coding	572	ccaggctcgaagggtact	0	298
	20626	Coding	578	tgttctccaggctcgaag	33	299
	20627	Coding	584	agatgatgttctccaggt	0	300
20	20628	Coding	591	atccggaagatgatgttc	0	301
	20629	Coding	624	ctccgctccgaccgctgg	56	302
	20630	Coding	634	gatccacttctccgctc	59	303
	20631	Coding	655	tgtcacgttctcaaagca	0	304
	20632	Coding	663	atgatggatgtcacgttc	0	305
25	20633	Coding	671	cgagaaacatgatggatg	0	306
	20634	Coding	682	gctgagggcgacgagaaa	75	307
	20635	Coding	709	cgactccaccaggacttg	40	308
	20636	Coding	726	atccggttctcgtgtcc	22	309
	20637	Coding	728	ccatccggttctcgttgt	19	310
30	20638	Coding	744	agggctttgctctcctcc	77	311
	20639	Coding	754	ggtccggaacagggtttt	26	312
	20640	Coding	766	gtaggtgatgatgtccg	0	313
	20641	Coding	787	ggaggagtcttggaacca	64	314
	20642	Coding	803	tgaggaagaggatgacgg	0	315
35	20643	Coding	818	gcaggtccttctgttga	6	316
	20644	Coding	831	atcttgtcctccagcagg	4	317
	20645	Coding	842	gcgagtacaggatcttgt	17	318
	20646	Coding	858	aagtagtcaccagggtgc	0	319
	20647	Coding	910	gatgaactcccgcccgcc	52	320
40	20648	Coding	935	ggttcaggtcacgaaca	71	321
	20649	Coding	958	gtagatgatcttgtcgtc	0	322
	20650	Coding	972	cacgtgaagtgtgagtag	0	323
	20651	Coding	993	atgttctccgtgtcggtg	0	324
	20652	Coding	1014	acggccgcgaacacgaag	6	325
45	20653	Coding	1027	gatggtgtccttcacggc	0	326
	20654	Coding	1043	tcaggttcagctgcagga	3	327
	20655	Coding	1059	accagattgtactcttc	0	328

EXAMPLE 28: Antisense inhibition of G-alpha-11 expression- phosphorothioate 2'-MOE gapmer oligonucleotides

In accordance with the present invention, a second series of oligonucleotides targeted to human G-alpha-11 were synthesized. The oligonucleotide sequences are shown in Table 17. Target sites are indicated by nucleotide numbers, as given in the sequence source reference (Genbank accession no. AF011497), to which the oligonucleotide binds.

All compounds in Table 17 are chimeric oligonucleotides ("gapmers") 18 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by four-nucleotide "wings". The wings are composed of 2'-methoxyethyl (2'-MOE)nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide. Cytidine residues in the 2'-MOE wings are 5-methylcytidines.

Data were obtained by real-time quantitative PCR as described in other examples herein and are averaged from three experiments. If present, "N.D." indicates "no data".

Table 17

Inhibition of G-alpha-11 mRNA levels by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap

	ISIS#	REGION	TARGET SITE	SEQUENCE	% Inhibition	SEQ ID NO.
20	20981	Coding	1	gatggactccagagtc	0	249
	20982	Coding	6	gccatgatggactccaga	0	250
	20983	Coding	9	cacgccatgatggactcc	0	251
25	20984	Coding	25	ctcatcgctcaggcaaca	0	252
	20985	Coding	31	cttcacctcatcgctcag	2	253
	20986	Coding	36	gactccttcacctcatcg	0	254
	20987	Coding	45	atccgcttgactccttc	19	255
	20988	Coding	50	cgttgatccgcttgact	15	256
30	20989	Coding	61	ctcgatctcggcgttgat	0	257
	20990	Coding	77	cccgccgcagctgcttct	41	258
	20991	Coding	106	cttgagctcgcgcgggc	19	259
	20992	Coding	116	gcagcagcagcttgagct	23	260
	20993	Coding	127	gcccgtgccgagcagcag	38	261
35	20994	Coding	146	acgtgcttctccgctct	34	262
	20995	Coding	159	atctgcttgatgaacgtg	56	263

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5	20996	Coding	162	cgcacatctgcttgatgaac	31	264
	20997	Coding	184	gtagccggcgccgtggat	0	265
	20998	Coding	197	tgtcctcctccgagtagc	42	266
	20999	Coding	199	cttgtctcctccgagta	0	267
	21000	Coding	207	aagccgcgcttgtctcc	73	268
10	21001	Coding	222	tagacgagcttggtgaag	0	269
	21002	Coding	230	tgttctggtagacgagct	61	270
	21003	Coding	242	tggcgggtgaagatgttct	14	271
	21004	Coding	258	cggatcatggcctgcatg	84	272
	21005	Coding	271	cgtctccatggcccggat	70	273
15	21006	Coding	285	tagaggatcttgagcgtc	39	274
	21007	Coding	287	tgtagaggatcttgagcg	28	275
	21008	Coding	297	tgctcgtacttgtagagg	70	276
	21009	Coding	306	gccttgttctgctcgtac	76	277
	21010	Coding	309	ttggccttgttctgctcg	0	278
20	21011	Coding	319	caggagcgcattggcctt	87	279
	21012	Coding	340	ctccacgtccacctccc	0	280
	21013	Coding	349	ggtcaccttctccacgtc	69	281
	21014	Coding	362	gatgctcgaaggtggtca	0	282
	21015	Coding	373	actgacgtactgatgctc	69	283
25	21016	Coding	382	cttgatggcactgacgta	32	284
	21017	Coding	388	cagggtcttgatggcact	19	285
	21018	Coding	409	ctggatgcccgggtcctc	63	286
	21019	Coding	411	tcttggatgcccgggtcc	56	287
	21020	Coding	429	cgcctgcggtcgtagcat	73	288
30	21021	Coding	440	gctggtagctgcgcctgc	68	289
	21022	Coding	459	tacttggcagagtcggag	50	290
	21023	Coding	468	gtcaggtagtacttggca	13	291
	21024	Coding	479	ggccaacgtcggtagggt	64	292
	21025	Coding	489	gtggcgatcgggtcaacg	52	293
35	21026	Coding	503	gcaggtagcccaagggtgg	52	294
	21027	Coding	518	cgtcctgctgggtgggca	0	295
	21028	Coding	544	ggtggtgggcacgcggac	81	296
	21029	Coding	555	tcgatgatgccggtggtg	48	297
	21030	Coding	572	ccaggctcgaagggtact	61	298
40	21031	Coding	578	tgttctccaggctcgaaag	0	299
	21032	Coding	584	agatgatgttctccaggt	0	300
	21033	Coding	591	atccggaagatgatgttc	0	301
	21034	Coding	624	ctccgctccgaccgtgg	59	302
	21035	Coding	634	gateccacttctccgctc	17	303
40	21036	Coding	655	tgtcacgttctcaaagca	9	304
	21037	Coding	663	atgatggatgtcacgttc	41	305
	21038	Coding	671	cgagaacatgatggatg	0	306

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	21039	Coding	682	gctgagggcgacgagaaa	11	307
	21040	Coding	709	cgactccaccaggacttg	0	308
	21041	Coding	726	atccggttctcgttgcc	67	309
	21042	Coding	728	ccatccggttctcgttgt	30	310
5	21043	Coding	744	agggctttgctctcctcc	61	311
	21044	Coding	754	gggccggaacagggttt	72	312
	21045	Coding	766	gtaggtgatgatggtccg	68	313
	21046	Coding	787	ggaggagtctggaacca	54	314
	21047	Coding	803	tgaggaaaggatgacgg	23	315
10	21048	Coding	818	gcaggtccttctgttgga	0	316
	21049	Coding	831	atctgtcctccagcagg	39	317
	21050	Coding	842	gcgagtacaggatcttgt	74	318
	21051	Coding	858	aagtagtccaccagggtgc	36	319
	21052	Coding	910	gatgaactcccgcgccgc	67	320
15	21053	Coding	935	gggtcagggtccacgaaca	37	321
	21054	Coding	958	gtatgatgatctgtcgct	64	322
	21055	Coding	972	cacgtgaagtgtgagtag	37	323
	21056	Coding	993	atgtctccgtgtcggtg	0	324
	21057	Coding	1014	acggccgcgaacacgaag	0	325
20	21058	Coding	1027	gatggtgtccttcacggc	69	326
	21059	Coding	1043	tcagggtcagctgcagga	0	327
	21060	Coding	1059	accagattgtactcttc	0	328

EXAMPLE 29: Automated Assay of AKT-1 Oligonucleotide Activity

25 Akt-1 (also known as PKB alpha and RAC-PK alpha) is a member of the AKT/PKB family of serine/threonine kinases and has been shown to be involved in a diverse set of signaling pathways.

Oligonucleotides were designed as described in Example 2, synthesized as described in Examples 3 through 8, analyzed as described in Examples 9 and assayed as described in Example 10 except for target specific primer and probes. AKT-1 probes and primers were designed to hybridize to the human AKT-1 sequence, using published sequence information (GenBank accession number M63167, incorporated herein by reference as SEQ ID NO:329). For Akt-1 the PCR primers were:

forward primer: CGTGACCATGAACGAGTTTGA (SEQ ID NO: 330)

35 reverse primer: CAGGATCACCTTGCCGAAA (SEQ ID NO: 331) and the PCR probe was: FAM-CTGAAGCTGCTGGGCAAGGGCA-TAMRA (SEQ ID NO: 332) where FAM (PE-Applied Biosystems, Foster City, CA) is the fluorescent reporter dye) and TAMRA (PE-Applied Biosystems, Foster City, CA) is the quencher dye.

EXMAMPLE 30: Antisense inhibition of Akt-1 expression- phosphorothioate oligodeoxynucleotides

In accordance with the present invention, a series of oligonucleotides were designed to target different regions of the human Akt-1 RNA, using published sequences (GenBank accession number M63167, incorporated herein by reference as SEQ ID NO: 329). The oligonucleotides are shown in Table 18. Target sites are indicated by nucleotide numbers, as given in the sequence source reference (Genbank accession no. M63167), to which the oligonucleotide binds. All compounds in Table 18 are oligodeoxynucleotides with phosphorothioate backbones (internucleoside linkages) throughout. The compounds were analyzed for effect on Akt-1 mRNA levels by quantitative real-time PCR as described in other examples herein. Data are averages from three experiments. If present, "N.D." indicates "no data".

Table 18

Inhibition of Akt-1 mRNA levels by phosphorothioate oligodeoxynucleotides

ISIS#	REGION	TARGET SITE	SEQUENCE	% Inhibition	SEQ ID NO.
28880	5' UTR	4	cctgtgccctgtcccag	55	333
28881	5' UTR	27	cctaagcccctggtgaca	15	334
28882	5' UTR	62	ctttgacttctttgaccc	68	335
28883	5' UTR	70	ggcagcccctttgacttc	53	336
28884	Coding	213	caaccctccttcacaata	24	337
28885	Coding	234	tactccccctgtttgtgc	0	338
28886	Coding	281	tgccatcattcttgagga	65	339
28887	Coding	293	agccaatgaaggtgcat	67	340
28888	Coding	352	cacagagaagttgttgag	22	341
28889	Coding	496	agtctggatggcggtgt	49	342
28890	Coding	531	tctcctcctcctgcttc	9	343
28891	Coding	570	cctgagttgtcactgggt	49	344
28892	Coding	666	ccgaaagtgcccttgccc	56	345
28893	Coding	744	gccacgatgacttccttc	60	346
28894	Coding	927	cggctctcggagaacaca	0	347
28895	Coding	990	acgttcttctccgagtgc	30	348
28896	Coding	1116	gtccgcgaaaaggctcttc	66	349
28897	Coding	1125	tactcaggtgtgccgcaa	66	350
28898	Coding	1461	ggcttgaagggtgggctg	41	351
28899	Coding	1497	tcaaaatacctgggtgtca	51	352

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	28900	Coding	1512	gccgtgaactcctcatca	56	353
	28901	Coding	1541	ggtcaggtgggtgatgg	0	354
	28902	Coding	1573	ctcgtgtccacacactc	61	355
	28903	3' UTR	1671	gcctctccatccctcaa	76	356
5	28904	3' UTR	1739	acagcgtggcttctctca	12	357
	28905	3' UTR	1814	ttttctccctaccccg	64	358
	28906	3' UTR	1819	gatagttttctccctac	0	359
	28907	3' UTR	1831	taaaacccgcaggatagt	74	360
	28908	3' UTR	1856	ggagaacaaactgatga	0	361
10	28909	3' UTR	1987	ctggctgacagagtggg	59	362
	28910	3' UTR	1991	gcggctggctgacagagt	61	363
	28911	3' UTR	2031	cccagagagatgacagat	46	364
	28912	3' UTR	2127	gctgctgtgtgcctgcca	38	365
	28913	3' UTR	2264	cataatacacaataacaa	39	366
15	28914	3' UTR	2274	atttgaacaacataatac	11	367
	28915	3' UTR	2397	aagtgtaccgtggagag	57	368
	28916	3' UTR	2407	cgaaaagggtcaagtgtcta	41	369
	28917	3' UTR	2453	caggagtcaggaggaggc	13	370
	28918	3' UTR	2545	aaagttgaatgttgtaaa	10	371
20	28919	3' UTR	2553	aaaataactaaagttgaat	25	372

EXAMPLE 31: Antisense inhibition of Akt-1 expression- phosphorothioate 2'-MOE gapmer oligonucleotides

In accordance with the present invention, a second series of oligonucleotides targeted to human Akt-1 were synthesized. The oligonucleotide sequences are shown in Table 19. Target sites are indicated by nucleotide numbers, as given in the sequence source reference (Genbank accession no. M63167), to which the oligonucleotide binds.

All compounds in Table 19 are chimeric oligonucleotides ("gapmers") 18 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by four-nucleotide "wings". The wings are composed of 2'-methoxyethyl (2'-MOE)nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide. Cytidine residues in the 2'-MOE wings are 5-methylcytidines.

Data were obtained by real-time quantitative PCR as described in other examples herein and are averaged from three experiments. If present, "N.D." indicates "no data".

Table 19

Inhibition of Akt-1 mRNA levels by chimeric phosphorothioate oligonucleotides

having 2'-MOE wings and a deoxy gap

	ISIS#	REGION	TARGET SITE	SEQUENCE	% Inhibition	SEQ ID NO.
5	28920	5' UTR	4	ccctgtgccctgtcccag	88	333
	28921	5' UTR	27	cctaagcccctggtgaca	44	334
	28922	5' UTR	62	ctttgactctcttgaccc	61	335
	28923	5' UTR	70	ggcagccccttgacttc	79	336
10	28924	Coding	213	caaccctccttcacaata	72	337
	28925	Coding	234	tactccctcgtttgtgc	39	338
	28926	Coding	281	tgccatcattcttgagga	73	339
	28927	Coding	293	agccaatgaaggtgcat	62	340
	28928	Coding	352	cacagagaagttgtgag	48	341
15	28929	Coding	496	agtctggatggcggtgt	43	342
	28930	Coding	531	tcctcctcctcctgcttc	49	343
	28931	Coding	570	cctgagttgtcactgggt	71	344
	28932	Coding	666	ccgaaagtgcccttgcgc	64	345
	28933	Coding	744	gccacgatgacttccttc	66	346
20	28934	Coding	927	cggctctcggagaacaca	77	347
	28935	Coding	990	acgttctctccgagtgc	89	348
	28936	Coding	1116	gtgccgcaaaaggctctc	61	349
	28937	Coding	1125	tactcaggtgtgccgcaa	74	350
	28938	Coding	1461	ggcttgaaggggtggctg	54	351
25	28939	Coding	1497	tcaaaatacctggtgtca	78	352
	28940	Coding	1512	gccgtgaactcctcatca	88	353
	28941	Coding	1541	ggtcaggtggtgtgatgg	71	354
	28942	Coding	1573	ctcgtgtccacacactc	83	355
	28943	3' UTR	1671	gcctctccatccctccaa	86	356
30	28944	3' UTR	1739	acagcgtggcttctctca	73	357
	28945	3' UTR	1814	ttttctccctaccccgcc	77	358
	28946	3' UTR	1819	gatagtcttctccctac	43	359
	28947	3' UTR	1831	taaaacccgcaggatagt	64	360
	28948	3' UTR	1856	ggagaacaaactggatga	70	361
35	28949	3' UTR	1987	ctggctgacagagtgagg	90	362
	28950	3' UTR	1991	gcggctggtgacagagt	82	363
	28951	3' UTR	2031	cccagagagatgacagat	53	364
	28952	3' UTR	2127	gtgctgtgtgcctgcca	80	365
	28953	3' UTR	2264	cataatacacaataacaa	48	366
40	28954	3' UTR	2274	atttgaacaacataatac	39	367
	28955	3' UTR	2397	aagtgtaccgtggagag	38	368
	28956	3' UTR	2407	cgaaaagggtcaagtgtcta	83	369

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28957	3' UTR	2453	cagggagtcagggagggc	59	370
28958	3' UTR	2545	aaagtgaatgttgtaa	25	371
28959	3' UTR	2553	aaaatactaaagtgaat	45	372

What is claimed is:

1. A method of defining a set of compounds that modulate the expression of a target nucleic acid sequence via binding of said compounds with said target nucleic acid sequence comprising generating a library of virtual compounds *in silico* according to defined criteria, and evaluating *in silico* the binding of said virtual compounds with said target nucleic acid according to defined criteria.
2. A method of defining a set of oligonucleotides that modulate the expression of a target nucleic acid sequence via binding of said oligonucleotides with said target nucleic acid sequence comprising generating *in silico* a plurality of virtual oligonucleotides according to defined criteria, and evaluating *in silico* the binding of said plurality of virtual oligonucleotides with said target nucleic acid according to defined criteria.
3. A method of defining a set of compounds that modulate the expression of a target nucleic acid sequence via binding of said compounds with said target nucleic acid comprising, generating *in silico* a library of virtual compounds according to defined criteria wherein said virtual compounds modulate the expression of said target nucleic acid sequence, and robotically synthesizing synthetic compounds corresponding to at least some of said virtual compounds.
4. A method of defining a set of compounds that modulate the expression of a target nucleic acid sequence via binding of said compounds with said target nucleic acid comprising generating *in silico* virtual compounds according to defined criteria wherein said virtual compounds modulate the expression of said target nucleic acid sequence, synthesizing synthetic compounds corresponding to at least some of said virtual compounds, and robotically assaying said synthetic compounds for one or more desired physical, chemical or biological properties.
5. A method of defining a set of compounds that modulate the expression of a target nucleic acid sequence via binding of said compounds with said target nucleic acid sequence comprising generating *in silico* a library of nucleobase sequences according to

defined criteria and evaluating *in silico* a plurality of virtual oligonucleotides having said nucleobase sequences according to defined criteria.

- 5 6. A method of defining a set of compounds that modulate the expression of a target nucleic acid sequence via binding of said compounds with said target nucleic acid sequence comprising evaluating *in silico* a plurality of virtual compounds according to defined criteria and robotically synthesizing a plurality of synthetic compounds corresponding to said plurality of virtual compounds.
- 10 7. A method of defining a set of compounds that modulate the expression of a target nucleic acid sequence via binding of said compounds with said target nucleic acid sequence comprising evaluating *in silico* a plurality of virtual compounds according to defined criteria and robotically assaying a plurality of synthetic compounds corresponding to at least some of said virtual compounds for one or more desired physical, chemical or
- 15 biological properties.
8. A method of defining a set of compounds that modulate the expression of a target nucleic acid sequence via binding of said compounds with said target nucleic acid sequence comprising generating a library of nucleobase sequences *in silico* according to
- 20 defined criteria and robotically synthesizing a plurality of synthetic compounds compounds having said nucleobase sequences.
9. A method of defining a set of compounds that modulate the expression of a target nucleic acid sequence via binding of said compounds with said target nucleic acid
- 25 sequence comprising robotically synthesizing a plurality of synthetic compounds and robotically assaying said plurality of synthetic compounds for one or more desired physical, chemical or biological properties.
10. A method of defining a set of compounds that modulate the expression of a target
- 30 nucleic acid sequence via binding of said compounds with said target nucleic acid sequence comprising generating a library of nucleobase sequences *in silico* according to

defined criteria and robotically assaying a plurality of synthetic compounds having at least some of said nucleobase sequences for one or more desired physical, chemical or biological properties.

5 11. A method of generating a set of oligonucleotides that modulate the expression of a target nucleic acid sequence via binding of said oligonucleotides with said target nucleic acid sequence, comprising the steps of:

(a) generating a library of nucleobase sequences *in silico* according to defined criteria;

10 (b) evaluating *in silico* a plurality of virtual oligonucleotides having the nucleobase sequences of (a) according to defined criteria; and

(c) robotically synthesizing a plurality of synthetic oligonucleotides corresponding to at least some of said virtual oligonucleotides.

15 12. A method of generating a set of oligonucleotides that modulate the expression of a target nucleic acid sequence via binding of said oligonucleotides with said target nucleic acid sequence, comprising the steps of:

(a) generating a library of nucleobase sequences *in silico* according to defined criteria;

20 (b) evaluating *in silico* a plurality of virtual oligonucleotides having the nucleobase sequences of (a) according to defined criteria; and

(c) robotically assaying a plurality of synthetic oligonucleotides corresponding to at least some of said virtual oligonucleotides for one or more desired physical, chemical or biological properties.

25 13. A method of generating a set of oligonucleotides that modulate the expression of a target nucleic acid sequence via binding of said oligonucleotides with said target nucleic acid sequence, comprising the steps of:

30 (a) generating a library of nucleobase sequences *in silico* according to defined criteria;

(b) robotically synthesizing a plurality of synthetic oligonucleotides having at least

some of said nucleobase sequences; and

(c) robotically assaying said plurality of synthetic oligonucleotides for one or more desired physical, chemical or biological properties.

5 14. A method of generating a set of oligonucleotides that modulate the expression of a target nucleic acid sequence via binding of said oligonucleotides with said target nucleic acid sequence, comprising the steps of:

(a) evaluating *in silico* a plurality of virtual oligonucleotides according to defined criteria;

10 (b) robotically synthesizing a plurality of synthetic oligonucleotides corresponding to at least some of said virtual oligonucleotides; and

(c) robotically assaying said plurality of synthetic oligonucleotides for one or more desired physical, chemical or biological properties.

15 15. A method of generating a set of oligonucleotides that modulate the expression of a target nucleic acid sequence via binding of said oligonucleotides with said target nucleic acid sequence, comprising the steps of:

(a) generating a library of nucleobase sequences *in silico* according to defined criteria;

20 (b) evaluating *in silico* a plurality of virtual oligonucleotides having the nucleobase sequences of (a) according to defined criteria;

(c) robotically synthesizing a plurality of synthetic oligonucleotides corresponding to at least some of said virtual oligonucleotides; and

25 (d) robotically assaying said plurality of synthetic oligonucleotides for one or more desired physical, chemical or biological properties.

16. A method of generating a set of oligonucleotides that modulate the expression of a target nucleic acid sequence via binding of said oligonucleotides with said target nucleic acid sequence, comprising the steps of:

30 (a) generating a library of nucleobase sequences *in silico* according to defined criteria;

- (b) choosing an oligonucleotide chemistry;
- (c) robotically synthesizing a set of synthetic oligonucleotides having said nucleobase sequences of step (a) and said oligonucleotide chemistry of step (b);
- (d) robotically assaying said set of synthetic oligonucleotides of step (c) for a physical, chemical or biological activity; and
- (e) selecting a subset of said set of synthetic oligonucleotides of step (c) having a desired level of physical, chemical or biological activity in order to generate said set of compounds.
17. A method of generating a set of oligonucleotides that modulate the expression of a target nucleic acid sequence via binding of said oligonucleotides with said target nucleic acid sequence, comprising the steps of:
- (a) generating a library of nucleobase sequences *in silico* according to defined criteria;
- (b) choosing an oligonucleotide chemistry;
- (c) evaluating *in silico* a plurality of virtual oligonucleotides having the nucleobase sequences of (a) and the oligonucleotide chemistry of (b) according to defined criteria, and selecting those having preferred characteristics, in order to generate a set of preferred nucleobase sequences;
- (d) robotically synthesizing a set of synthetic oligonucleotides having said preferred nucleobase sequences of step (c) and said oligonucleotide chemistry of step (b);
- (e) robotically assaying said set of synthetic oligonucleotides of step (d) for a physical, chemical or biological activity; and
- (f) selecting a subset of said set of synthetic oligonucleotides of step (d) having a desired level of physical, chemical or biological activity in order to generate said set of oligonucleotides.

18. The method of claim 12, wherein said step of robotically assaying said plurality of synthetic oligonucleotide compounds is performed by computer-controlled real-time polymerase chain reaction or by computer-controlled enzyme-linked immunosorbent assay.

19. The method of claim 11, wherein said target nucleic acid sequence is that of a genomic DNA, a cDNA, a product of a polymerase chain reaction, an expressed sequence tag, an mRNA or a structural RNA.

5 20. The method of claim 11, wherein said target nucleic acid sequence is a human nucleic acid.

21. A method of identifying one or more nucleic acid sequences amenable to antisense binding of an oligonucleotide to said nucleic acid sequences comprising generating a
10 library of antisense nucleobase sequences *in silico* according to defined criteria.

22. A method of identifying a set of compounds that modulate the expression of a target nucleic acid sequence via binding of said compounds with said target nucleic acid sequence comprising evaluating *in silico* a plurality of virtual oligonucleotides according
15 to defined criteria.

23. A method of identifying one or more nucleic acid sequences amenable to antisense binding of an compound to said nucleic acid sequences comprising robotically synthesizing a plurality of synthetic antisense compounds.
20

24. A method of identifying one or more nucleic acid sequences amenable to antisense binding of an compound to said nucleic acid sequences comprising robotically assaying a plurality of synthetic antisense compounds for one or more desired physical, chemical or biological properties.
25

25. A method of identifying one or more nucleic acid sequences amenable to antisense binding of an oligonucleotide to said nucleic acid sequences comprising generating *in silico* a library of nucleobase sequences according to defined criteria and evaluating *in silico* a plurality of virtual oligonucleotides having said nucleobase sequences according to
30 defined criteria.

26. A method of identifying one or more nucleic acid sequences amenable to antisense binding of an oligonucleotide to said nucleic acid sequences comprising evaluating *in silico* a plurality of virtual oligonucleotides according to defined criteria and robotically synthesizing a plurality of synthetic oligonucleotides corresponding to least some of said virtual oligonucleotides.

27. A method of identifying one or more nucleic acid sequences amenable to antisense binding of an oligonucleotide to said nucleic acid sequences comprising evaluating *in silico* a plurality of virtual oligonucleotides according to defined criteria and robotically assaying a plurality of synthetic oligonucleotides corresponding to least some of said virtual oligonucleotides for one or more desired physical, chemical or biological properties.

28. A method of identifying one or more nucleic acid sequences amenable to antisense binding of an oligonucleotide to said nucleic acid sequences comprising generating a library of nucleobase sequences *in silico* according to defined criteria and robotically synthesizing a plurality of synthetic oligonucleotides having said nucleobase sequences.

29. A method of identifying one or more nucleic acid sequences amenable to antisense binding of an oligonucleotide to said nucleic acid sequences comprising robotically synthesizing a plurality of synthetic oligonucleotides and robotically assaying said plurality of synthetic oligonucleotides for one or more desired physical, chemical or biological properties.

30. A method of identifying one or more nucleic acid sequences amenable to antisense binding of an oligonucleotide to said nucleic acid sequences comprising generating a library of nucleobase sequences *in silico* according to defined criteria and robotically assaying a plurality of synthetic oligonucleotides having said nucleobase sequences for one or more desired physical, chemical or biological properties.

31. A method of identifying one or more nucleic acid sequences amenable to antisense

binding of an oligonucleotide to said nucleic acid sequences comprising the steps of:

(a) generating a library of nucleobase sequences *in silico* according to defined criteria;

5 (b) evaluating *in silico* a plurality of virtual oligonucleotides having the nucleobase sequences of (a) according to defined criteria; and

(c) robotically synthesizing a plurality of synthetic oligonucleotides corresponding to at least some of said virtual oligonucleotides.

32. A method of identifying one or more nucleic acid sequences amenable to antisense
10 binding of an oligonucleotide to said nucleic acid sequences, comprising the steps of:

(a) generating a library of nucleobase sequences *in silico* according to defined criteria;

(b) evaluating *in silico* a plurality of virtual oligonucleotides having the nucleobase sequences of (a) according to defined criteria; and

15 (c) robotically assaying a plurality of synthetic oligonucleotides corresponding to at least some of said virtual oligonucleotides for one or more desired physical, chemical or biological properties.

33. A method of identifying one or more nucleic acid sequences amenable to antisense
20 binding of an oligonucleotide to said nucleic acid sequences, comprising the steps of:

(a) generating a library of nucleobase sequences *in silico* according to defined criteria;

(b) robotically synthesizing a plurality of synthetic oligonucleotides having at least some of said nucleobase sequences; and

25 (c) robotically assaying said plurality of synthetic oligonucleotides for one or more desired physical, chemical or biological properties.

34. A method of identifying one or more nucleic acid sequences amenable to antisense
binding of an oligonucleotide to said nucleic acid sequences, comprising the steps of:

30 (a) evaluating *in silico* a plurality of virtual oligonucleotides according to defined criteria;

(b) robotically synthesizing a plurality of synthetic oligonucleotides corresponding to at least some of said virtual oligonucleotides; and

(c) robotically assaying said plurality of synthetic oligonucleotides for one or more desired physical, chemical or biological properties.

5

35. A method of identifying one or more nucleic acid sequences amenable to antisense binding of an oligonucleotide to said nucleic acid sequences, comprising the steps of:

(a) generating a library of nucleobase sequences *in silico* according to defined criteria;

10

(b) evaluating *in silico* a plurality of virtual oligonucleotides having the nucleobase sequences of (a) according to defined criteria;

(c) robotically synthesizing a plurality of synthetic oligonucleotides corresponding to at least some of said plurality of virtual oligonucleotides; and

(d) robotically assaying said plurality of synthetic oligonucleotides for one or more desired physical, chemical or biological properties.

15

36. A method of identifying one or more nucleic acid sequences amenable to antisense binding of an oligonucleotide to said nucleic acid sequences, comprising the steps of:

(a) generating a library of nucleobase sequences *in silico* according to defined criteria;

20

(b) choosing an oligonucleotide chemistry;

(c) robotically synthesizing a set of synthetic oligonucleotides having said nucleobase sequences of step (a) and said oligonucleotide chemistry of step (b);

(d) robotically assaying said set of synthetic oligonucleotides of step (c) for a physical, chemical or biological activity; and

25

(e) selecting a subset of said set of synthetic oligonucleotides of step (c) having a desired level of physical, chemical or biological activity.

37. A method of identifying one or more nucleic acid sequences amenable to antisense binding of an oligonucleotide to said nucleic acid sequences, comprising the steps of:

30

(a) generating a library of nucleobase sequences *in silico* according to defined

criteria;

(b) choosing an oligonucleotide chemistry;

(c) evaluating *in silico* a plurality of virtual oligonucleotides having the nucleobase sequences of (a) according to defined criteria, and selecting those having preferred characteristics, in order to generate a set of preferred nucleobase sequences;

(d) robotically synthesizing a set of synthetic oligonucleotides having said preferred nucleobase sequences of step (b) and said oligonucleotide chemistry of step (c);

(e) robotically assaying said set of synthetic oligonucleotides of step (d) for a physical, chemical or biological activity; and

(f) selecting a subset of said set of oligonucleotides of step (d) having a desired level of physical, chemical or biological activity.

38. The method of claim 32, wherein said step of robotically assaying said plurality of synthetic antisense oligonucleotides is performed by computer-controlled real-time polymerase chain reaction or by computer-controlled enzyme-linked immunosorbent assay.

39. The method of claim 31, wherein said nucleic acid sequence is that of a genomic DNA, a cDNA, a product of a polymerase chain reaction, an expressed sequence tag, an mRNA or a structural RNA.

40. The method of claim 31, wherein said nucleic acid sequence is a human nucleic acid.

41. A computer formatted medium comprising computer readable instructions for identifying compounds that have one or more desired properties according to defined criteria and that bind to a genomic DNA, a cDNA, a product of a polymerase chain reaction, an expressed sequence tag, an mRNA or a structural RNA.

42. A computer formatted medium comprising computer readable instructions for performing the method of any one of claims 1 to 20.

43. A computer formatted medium comprising computer readable instructions for performing a method of identifying one or more nucleic acid sequences amenable to antisense binding of a compound to said nucleic acid sequences.

5 44. A computer formatted medium comprising computer readable instructions for performing the method of any one of claims 21 to 40.

45. A computer formatted medium comprising one or more nucleic acid sequences amenable to antisense binding of a compound to said nucleic acid sequences in computer
10 readable form.

46. A computer formatted medium comprising one or more nucleic acid sequences amenable to antisense binding of a compound to said nucleic acid sequences in computer readable form, wherein said one or more nucleic acid sequences is identified according to
15 the method of any one of claims 21-40.

47. A process for validating the function of a gene or the product of said gene comprising generating *in silico* a library of nucleobase sequences targeted to said gene and robotically assaying a plurality of synthetic compounds having at least some of said
20 nucleobase sequences for effects on biological function.

48. A process for validating the function of a gene or the product of said gene, comprising the steps of:

- 25 (a) generating a library of nucleobase sequences *in silico* according to defined criteria;
- (b) evaluating *in silico* a plurality of virtual oligonucleotides having the nucleobase sequences of (a) according to defined criteria; and
- (c) robotically synthesizing a plurality of synthetic oligonucleotides corresponding to at least some of said virtual oligonucleotides.

30

49. A process for validating the function of a gene or the product of said gene,

comprising the steps of:

(a) generating a library of nucleobase sequences *in silico* according to defined criteria;

5 (b) evaluating *in silico* a plurality of virtual oligonucleotides having the nucleobase sequences of (a) according to defined criteria; and

(c) robotically assaying a plurality of synthetic oligonucleotides corresponding to at least some of said virtual oligonucleotides for effects on biological function.

10 50. A process for validating the function of a gene or the product of said gene, comprising the steps of:

(a) generating a library of nucleobase sequences *in silico* according to defined criteria;

(b) robotically synthesizing a plurality of synthetic oligonucleotides having at least some of said nucleobase sequences; and

15 (c) robotically assaying said plurality of synthetic oligonucleotides for effects on biological function.

51. A process for validating the function of a gene or the product of said gene, comprising the steps of:

20 (a) evaluating *in silico* a plurality of virtual oligonucleotides according to defined criteria;

(b) robotically synthesizing a plurality of synthetic oligonucleotides corresponding to at least some of said virtual oligonucleotides; and

25 (c) robotically assaying said plurality of synthetic oligonucleotides for effects on biological function.

52. A process for validating the function of a gene or the product of said gene, comprising the steps of:

30 (a) generating a library of nucleobase sequences *in silico* according to defined criteria;

(b) evaluating *in silico* a plurality of virtual oligonucleotides having the nucleobase

sequences of (a) according to defined criteria;

(c) robotically synthesizing a plurality of synthetic oligonucleotides corresponding to at least some of said virtual oligonucleotides; and

5 (d) robotically assaying said plurality of synthetic oligonucleotides for effects on biological function.

53. A process for validating the function of a gene or the product of said gene, comprising the steps of:

10 (a) generating a library of nucleobase sequences *in silico* according to defined criteria;

(b) choosing an oligonucleotide chemistry;

(c) robotically synthesizing a set of synthetic oligonucleotides having said nucleobase sequences of step (a) and said oligonucleotide chemistry of step (b);

15 (d) robotically assaying said set of synthetic oligonucleotides of step (c) for effects on biological function; and

(e) selecting a subset of said set of synthetic oligonucleotides of step (c) having a desired level of physical, chemical or biological activity in order to generate said set of compounds.

20 54. A process for validating the function of a gene or the product of said gene, comprising the steps of:

(a) generating a library of nucleobase sequences *in silico* according to defined criteria;

(b) choosing an oligonucleotide chemistry;

25 (c) evaluating *in silico* a plurality of virtual oligonucleotides having the nucleobase sequences of (a) and the oligonucleotide chemistry of (b) according to defined criteria, and selecting those having preferred characteristics, in order to generate a set of preferred nucleobase sequences;

(d) robotically synthesizing a set of synthetic oligonucleotides having said

30 preferred nucleobase sequences of step (c) and said oligonucleotide chemistry of step (b);

(e) robotically assaying said set of synthetic oligonucleotides of step (d) for effects

on biological function; and

(f) selecting a subset of said set of synthetic oligonucleotides of step (d) having a desired level of physical, chemical or biological activity in order to generate said set of oligonucleotides.

ABSTRACT

Iterative, preferably computer based iterative processes for generating synthetic compounds with desired physical, chemical and/or bioactive properties, i.e., active compounds, are provided. During iterations of the processes, a target nucleic acid sequence is provided or selected, and a library of candidate nucleobase sequences is generated *in silico* according to defined criteria. A "virtual" oligonucleotide chemistry is chosen and a library of virtual oligonucleotide compounds having the selected nucleobase sequences is generated. These virtual compounds are reviewed and compounds predicted to have particular properties are selected. The selected compounds are robotically synthesized and are preferably robotically assayed for a desired physical, chemical or biological activity. Active compounds are thus generated and, at the same time, preferred sequences and regions of the target nucleic acid that are amenable to oligonucleotide or sequence-based modulation are identified.

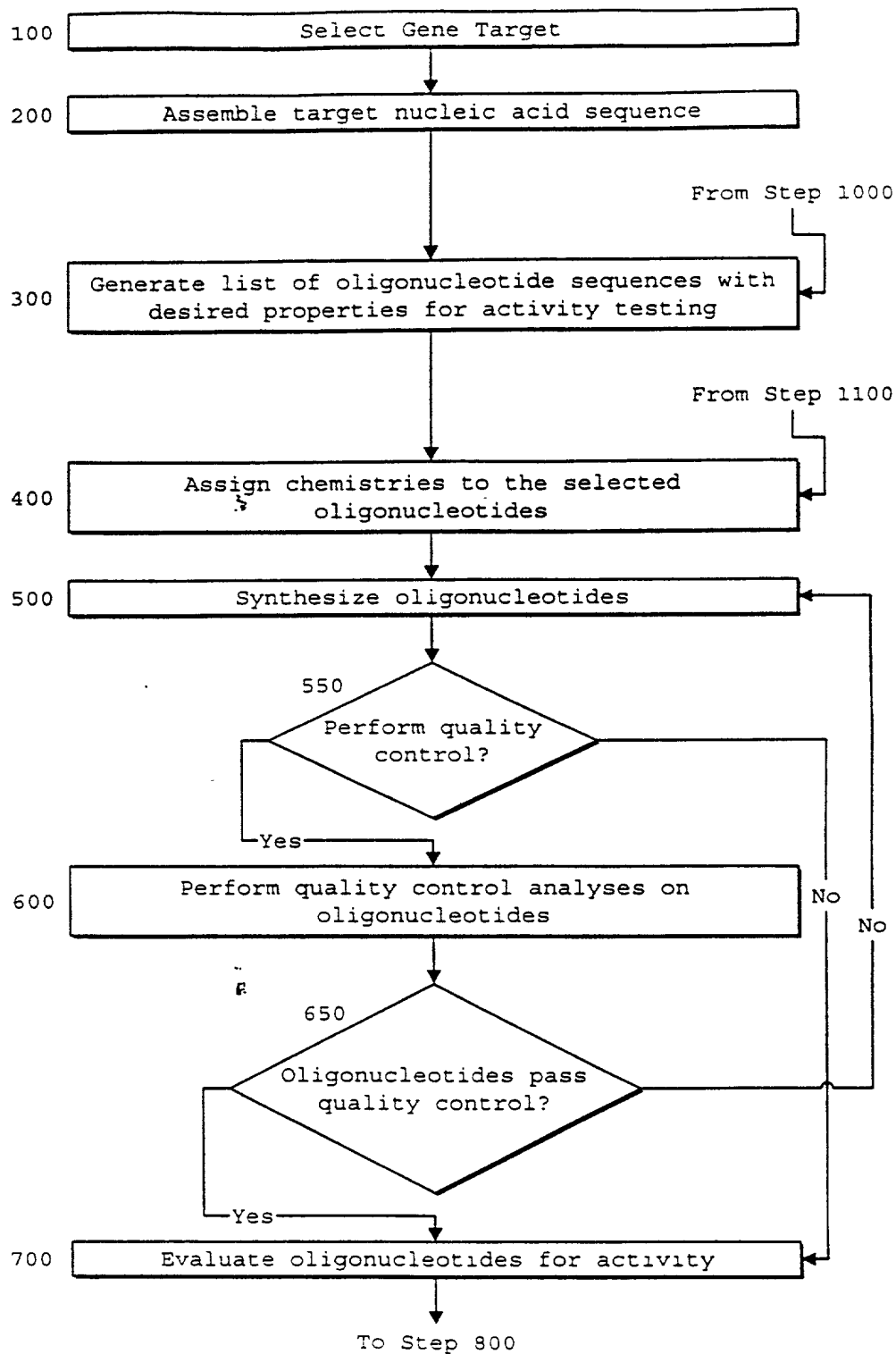


Figure 1

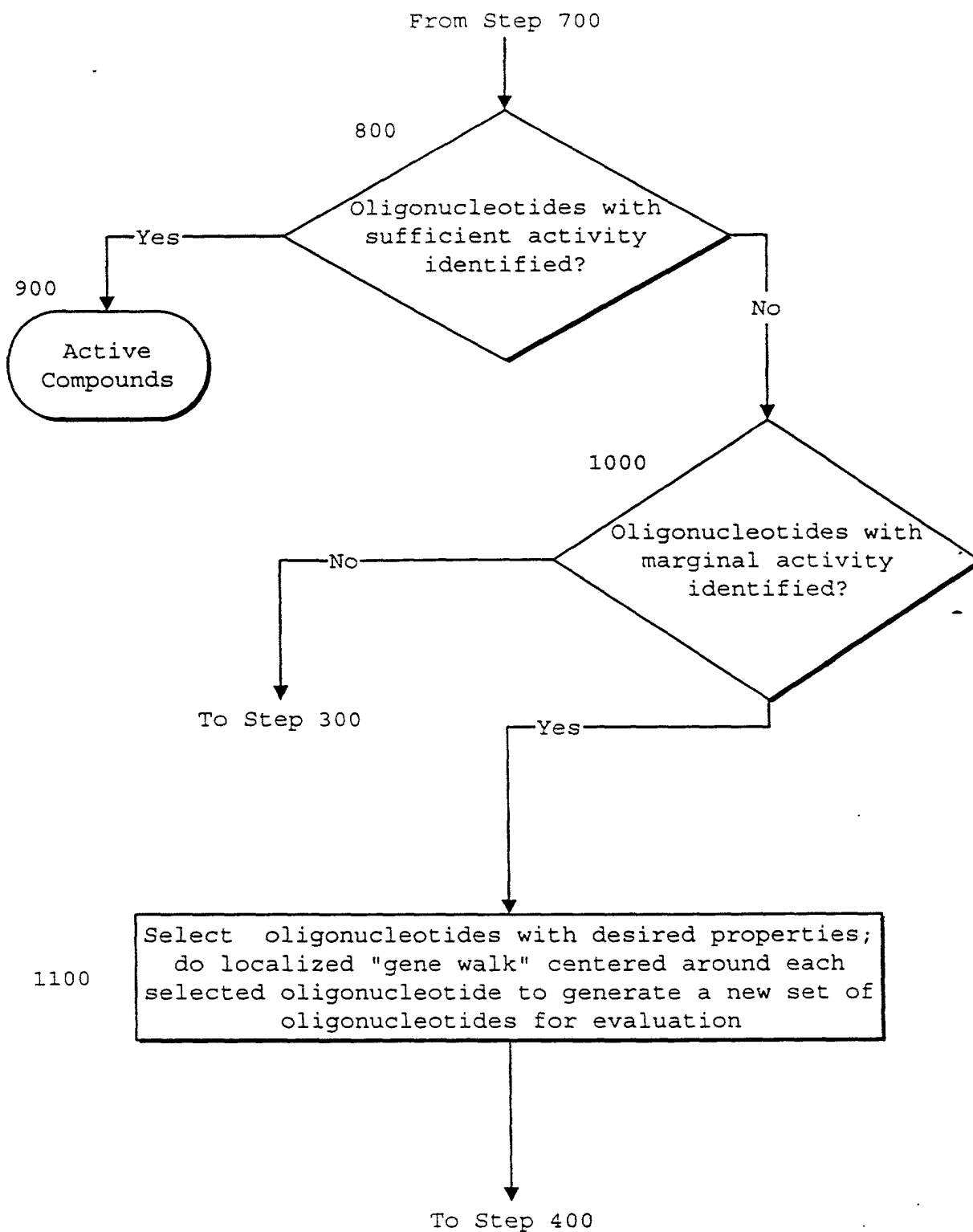


Figure 2

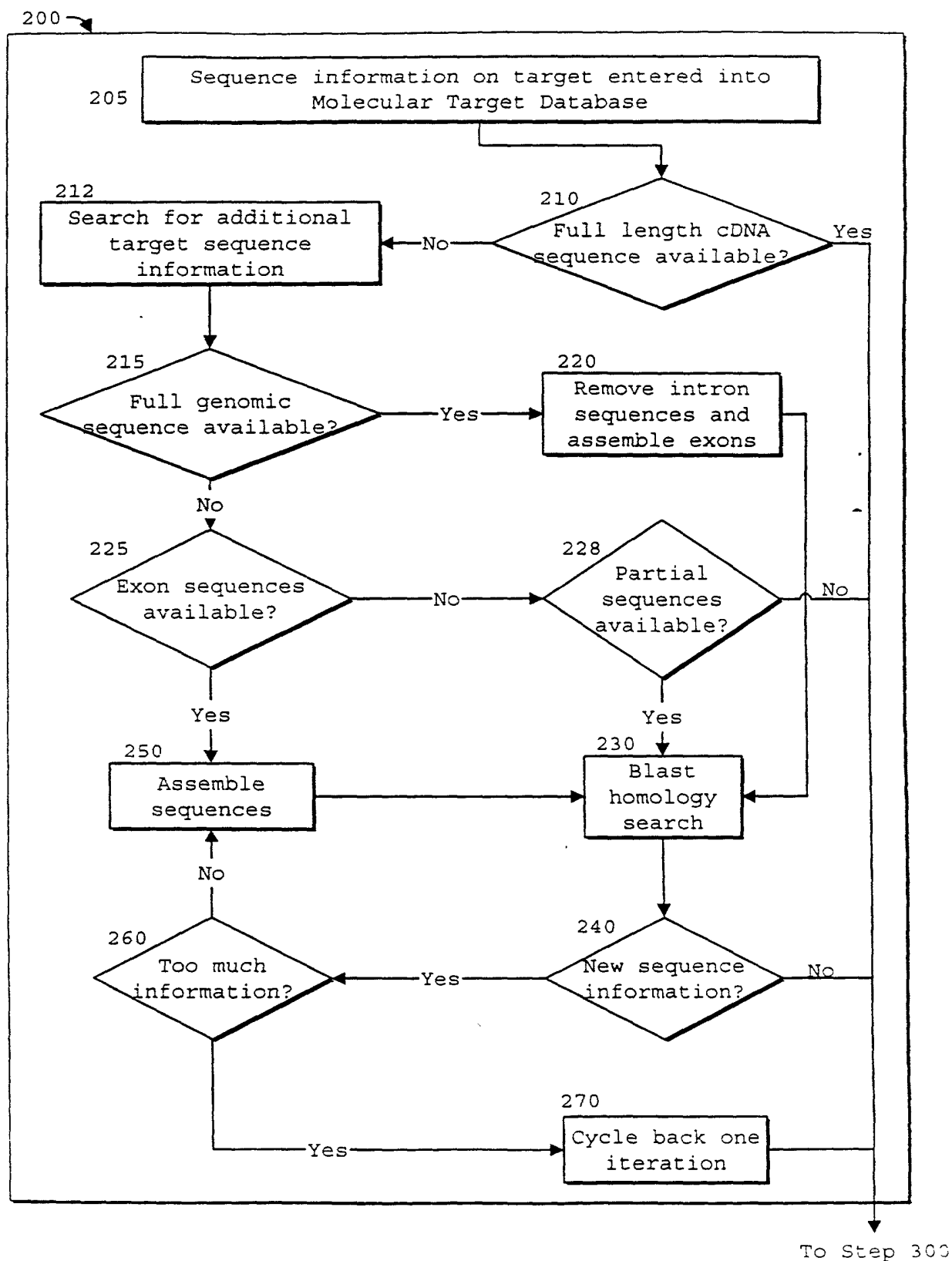


Figure 3

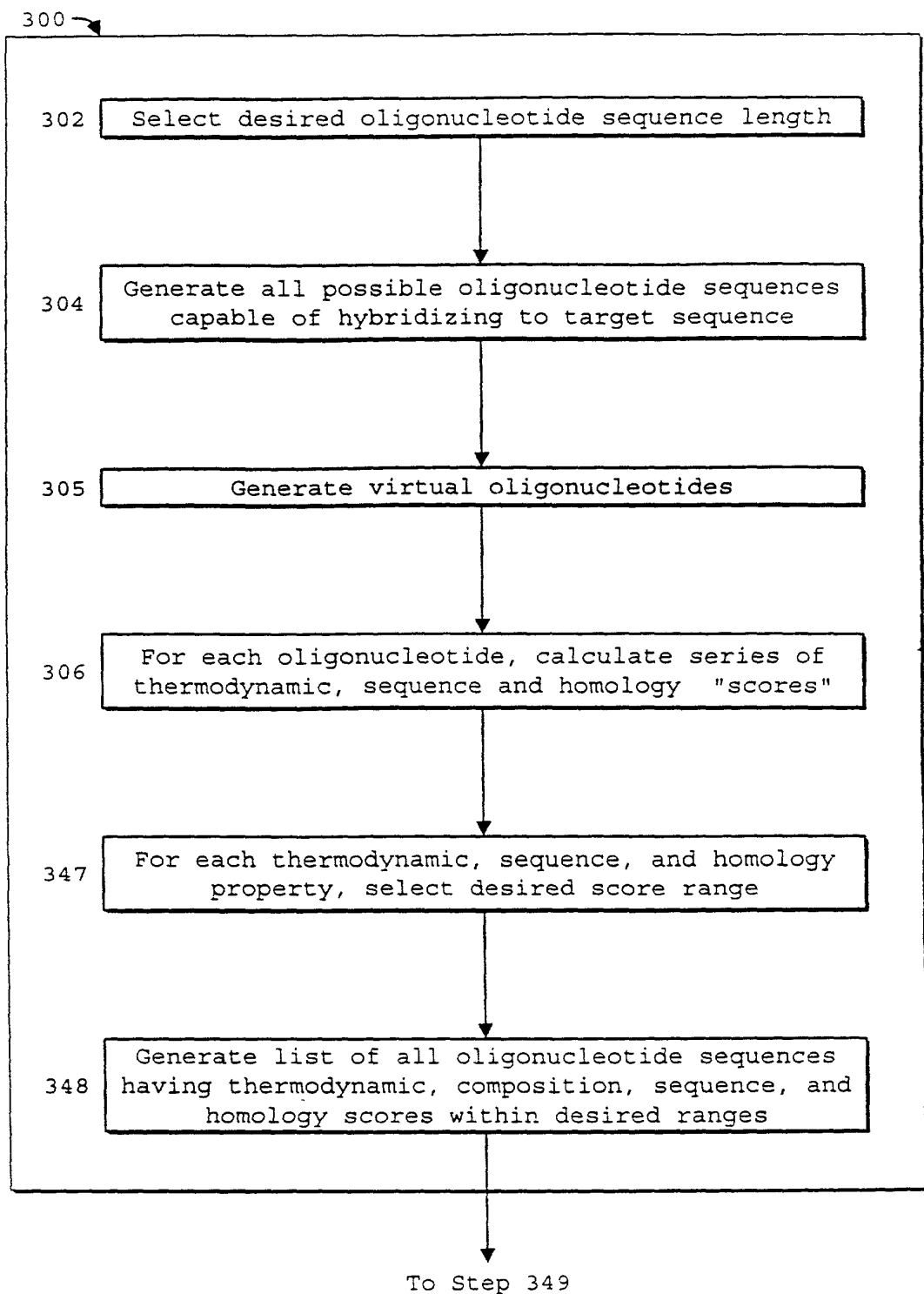


Figure 4

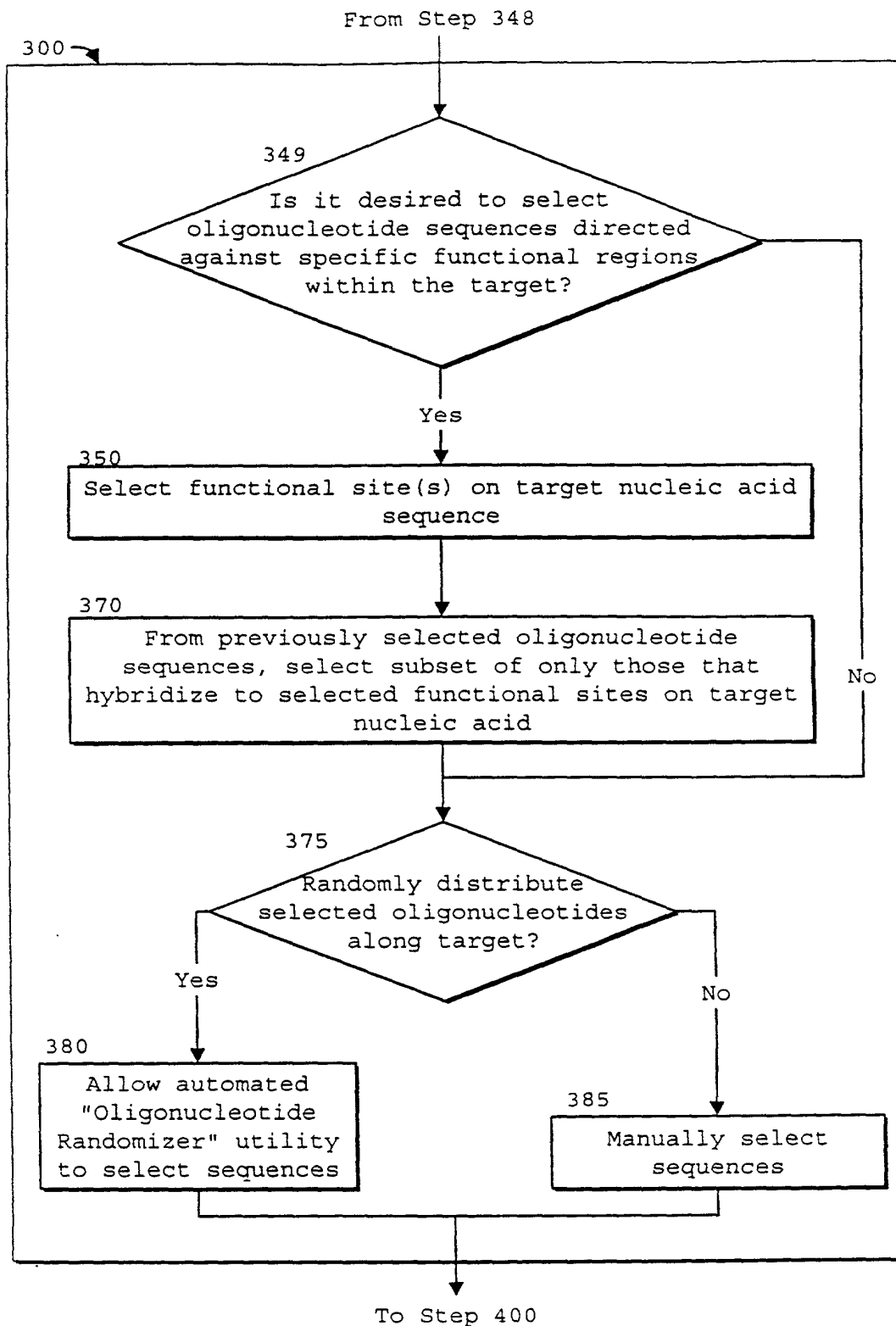


Figure 5

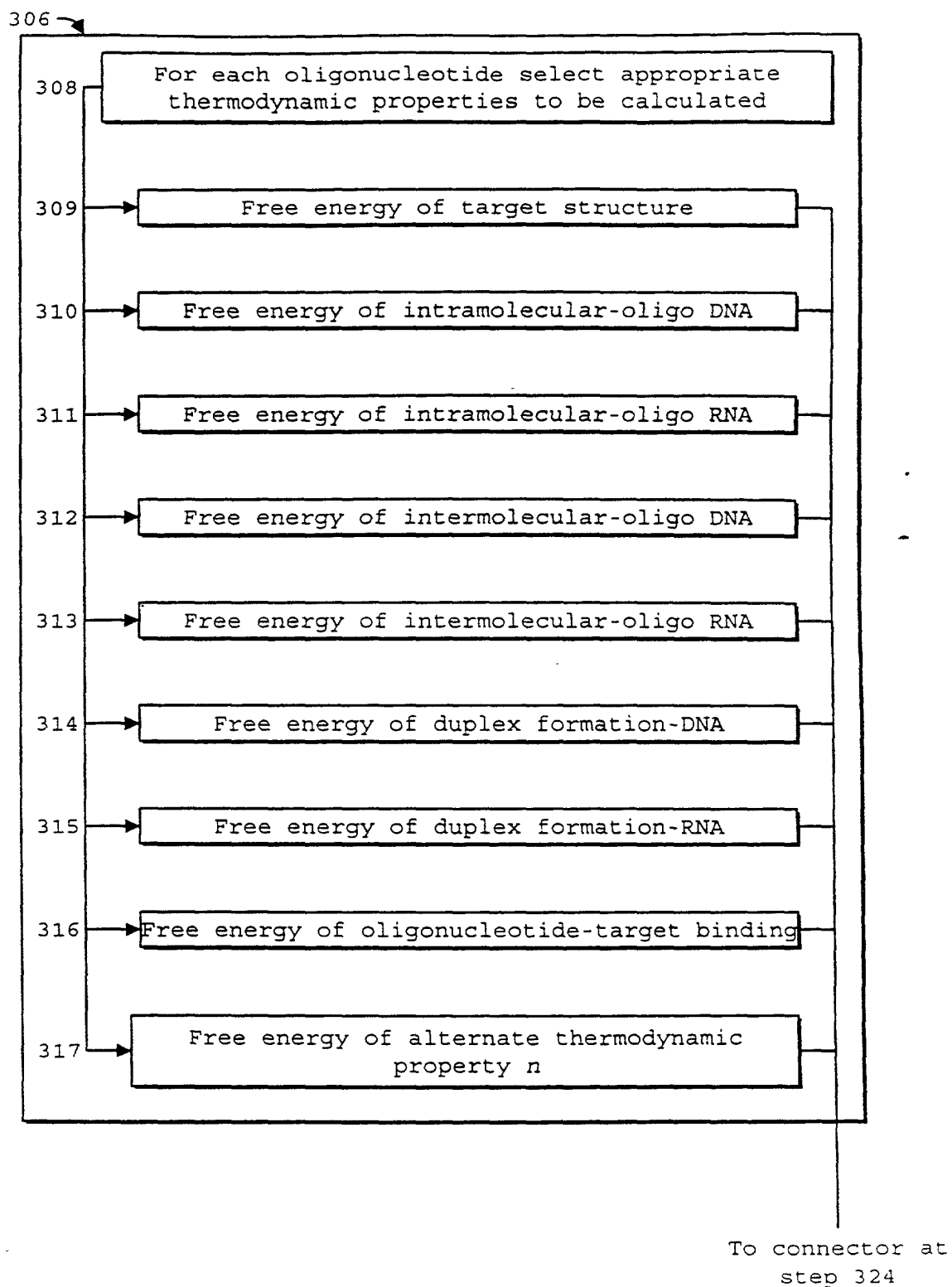
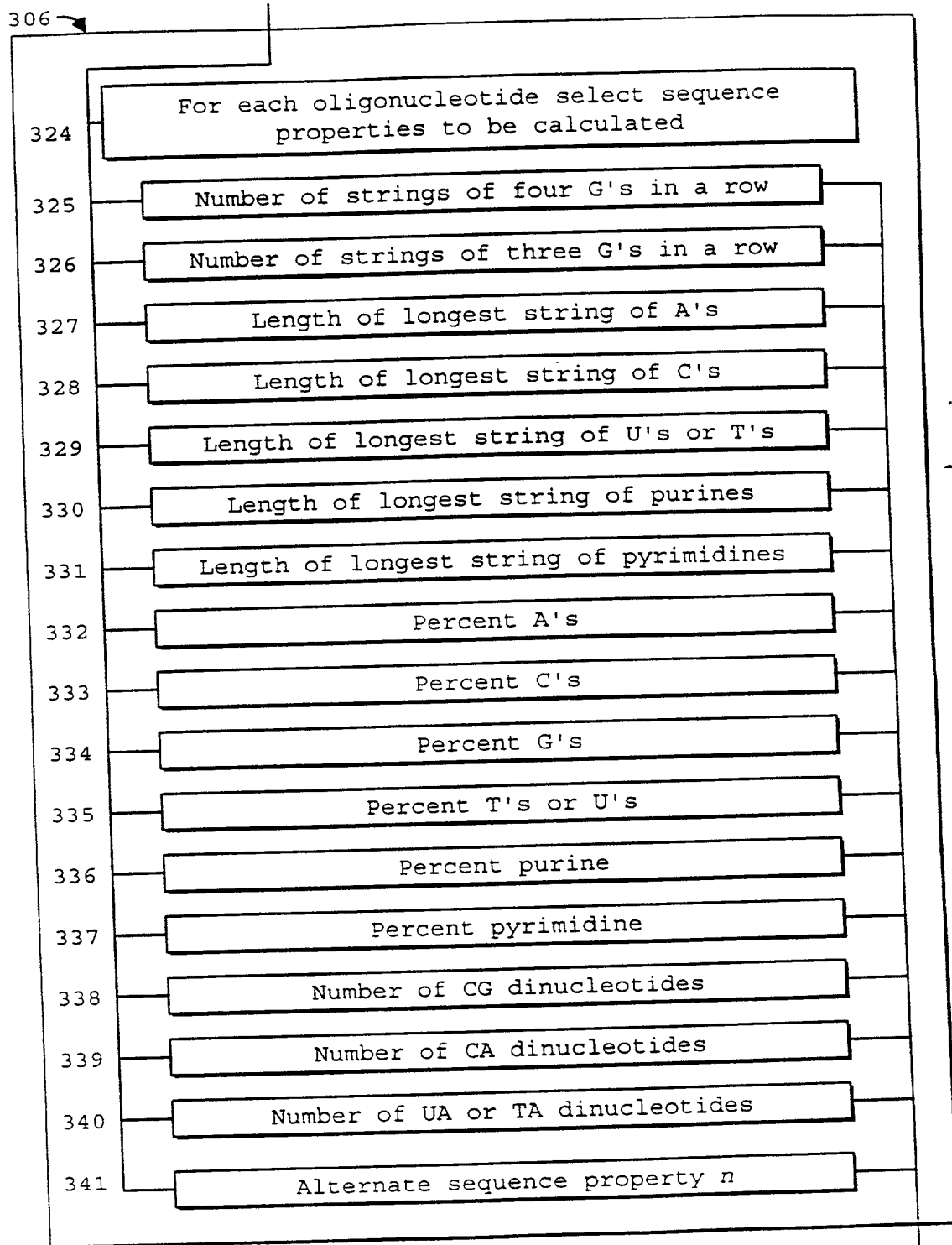


Figure 6

From connector
at step 317



To connector at
step 342

Figure 7

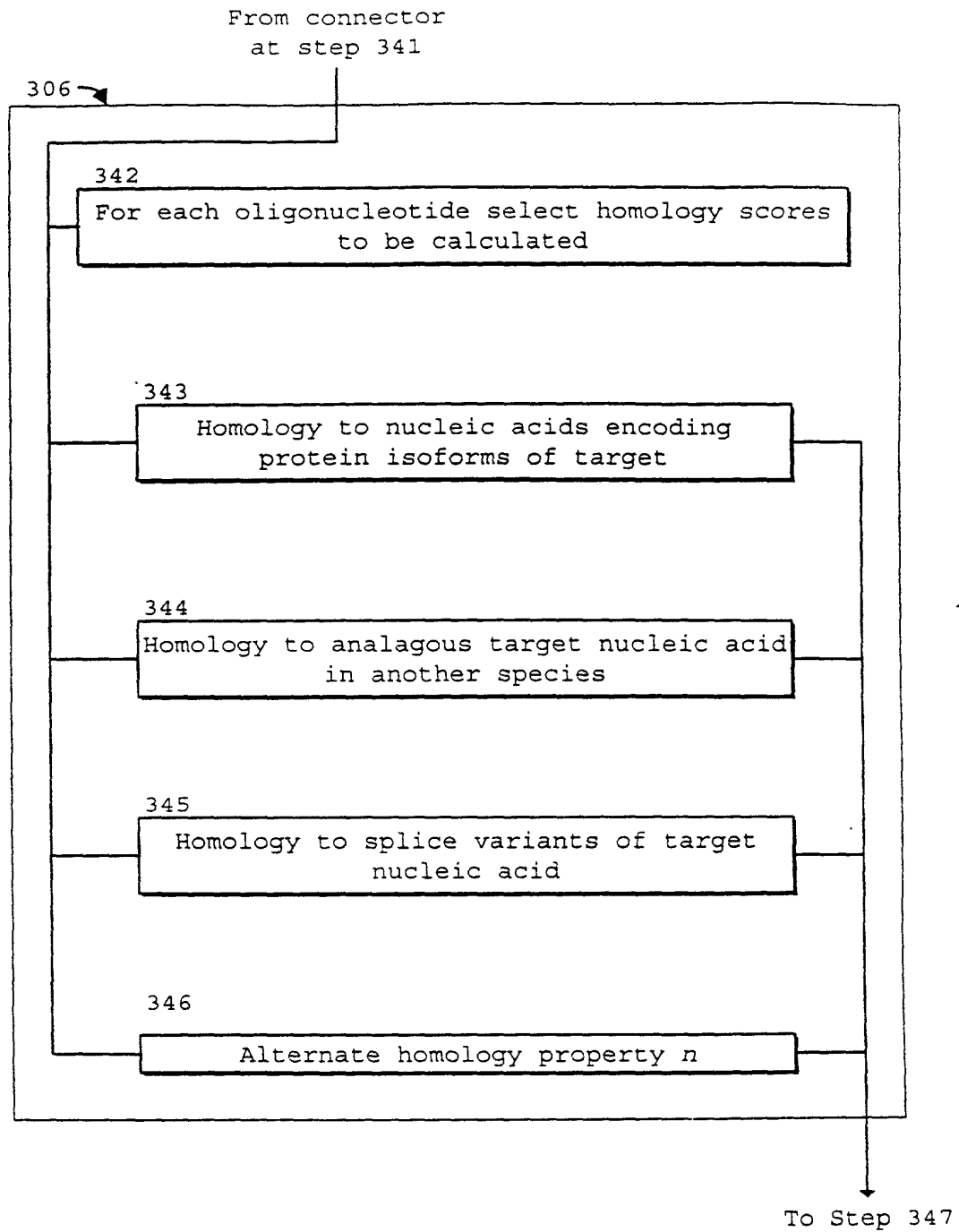
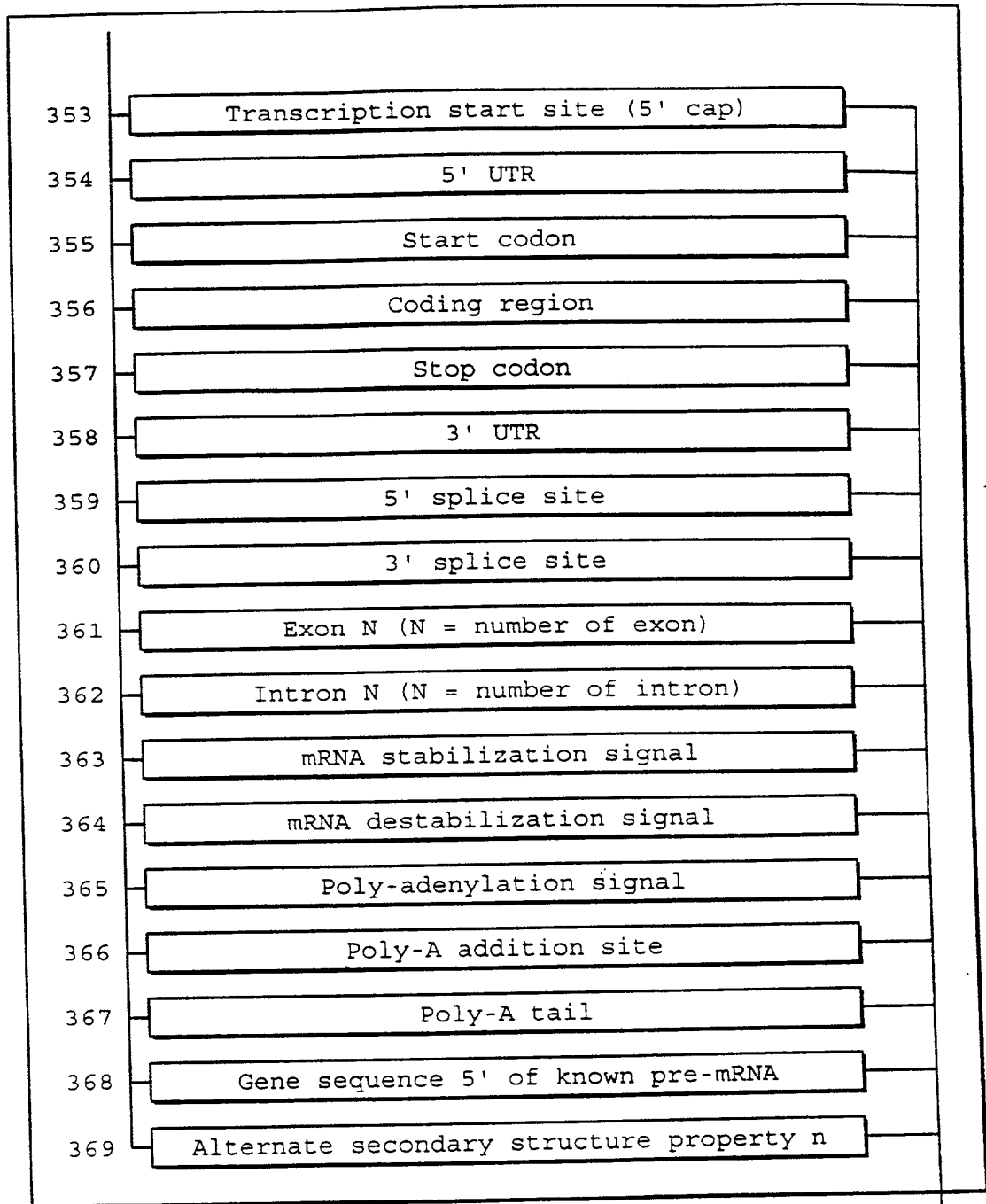


Figure 8

350



To Step 370

Figure 9

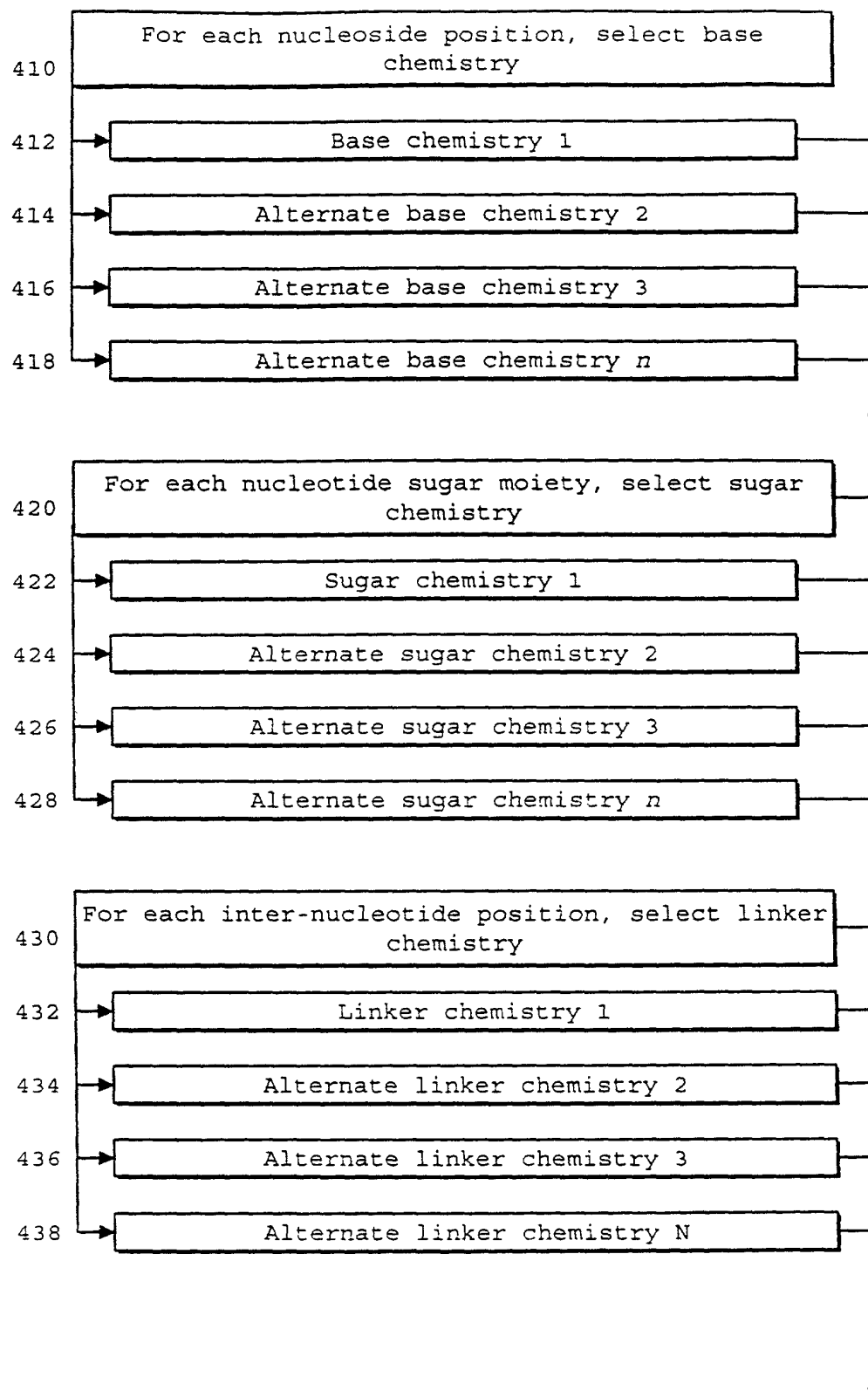


Figure 10

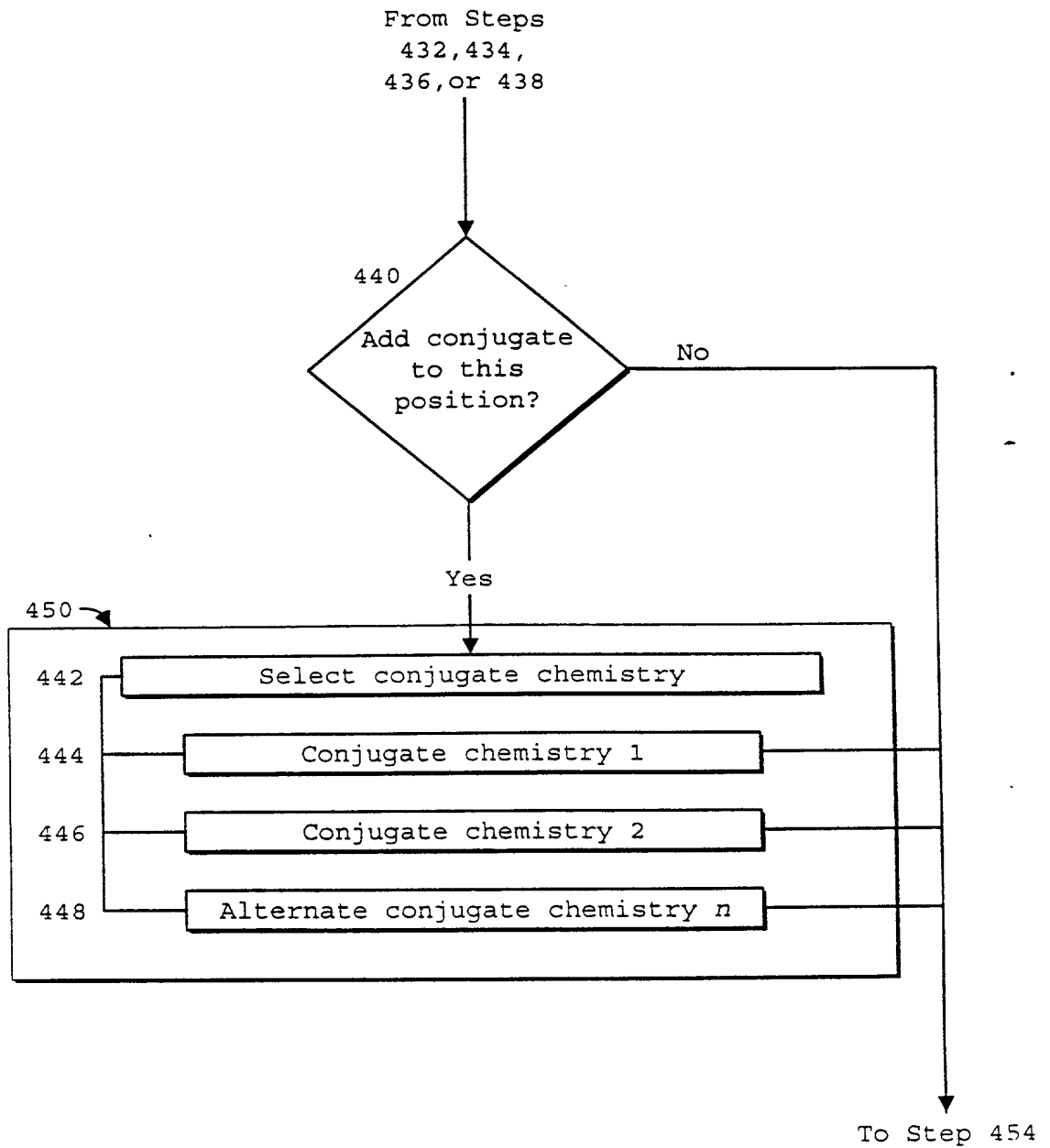


Figure 11

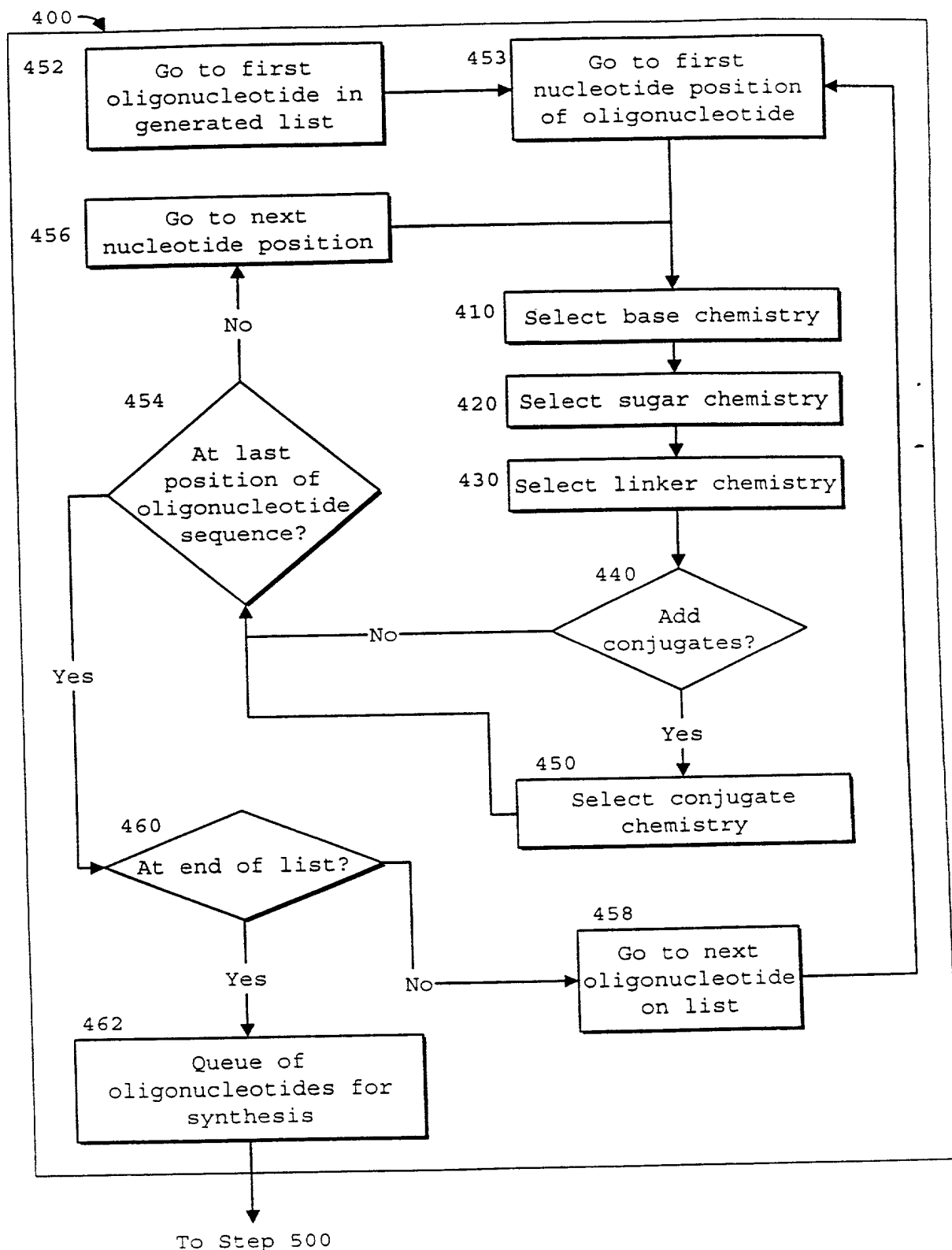


Figure 12

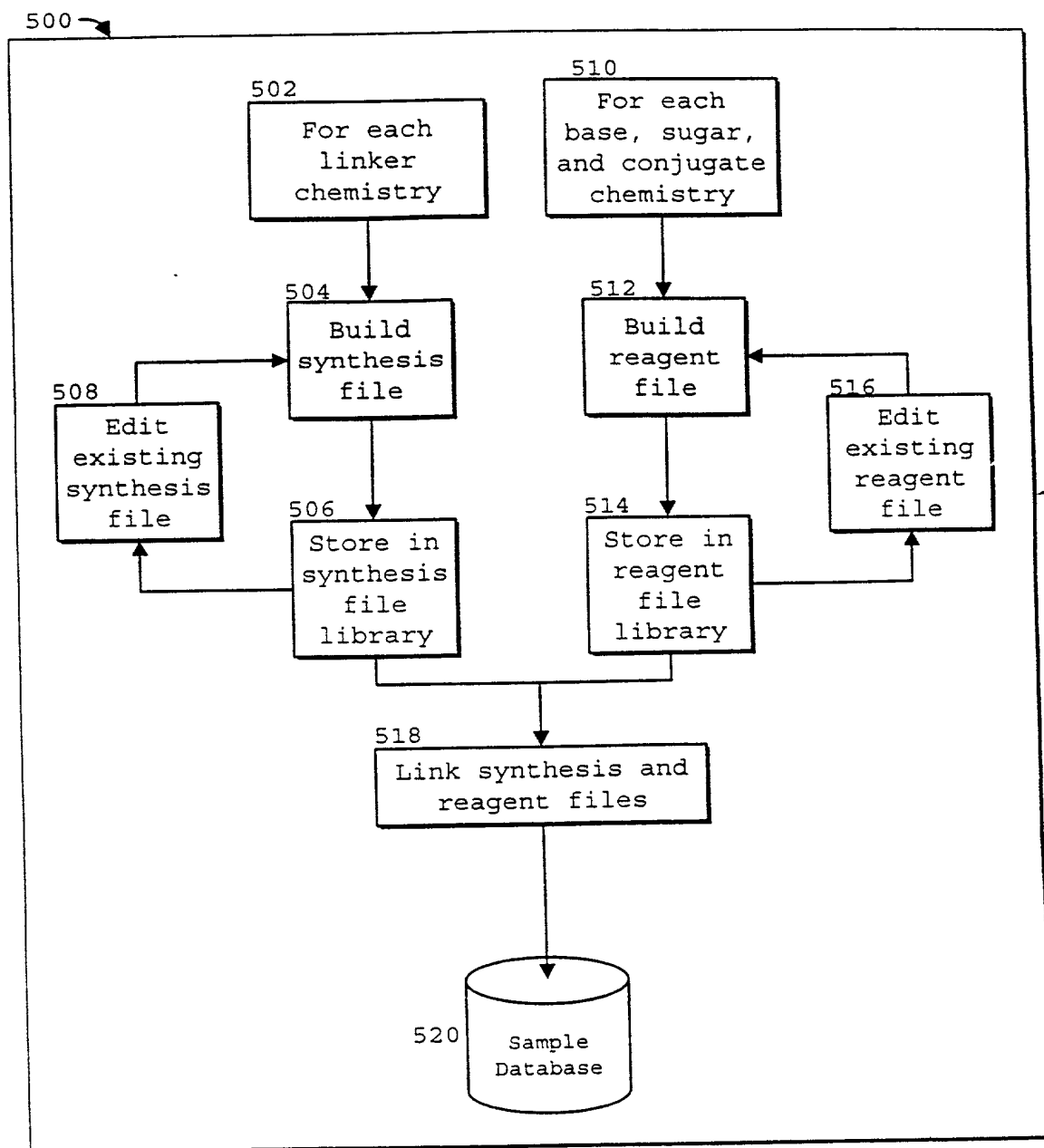


Figure 13

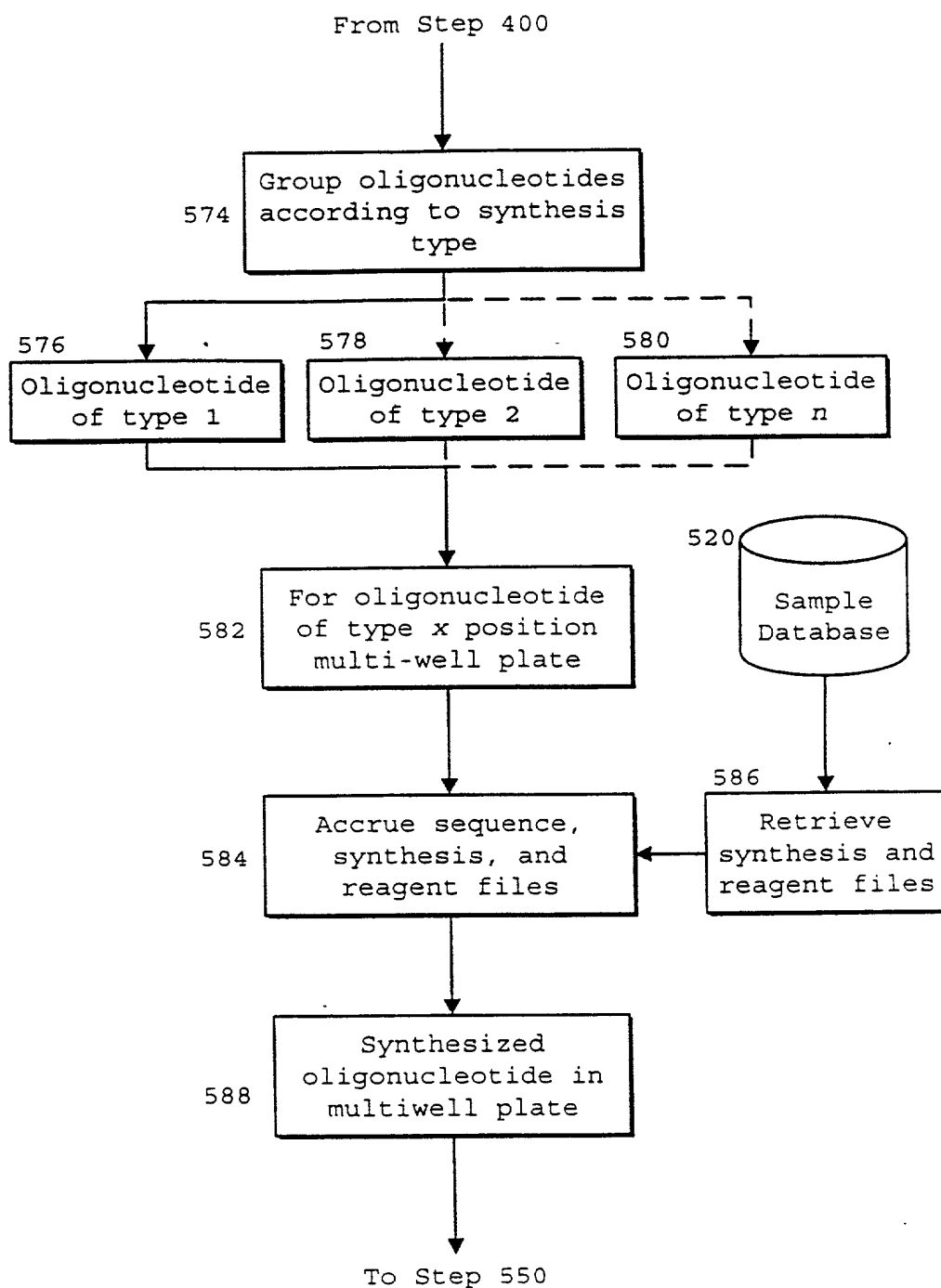


Figure 14

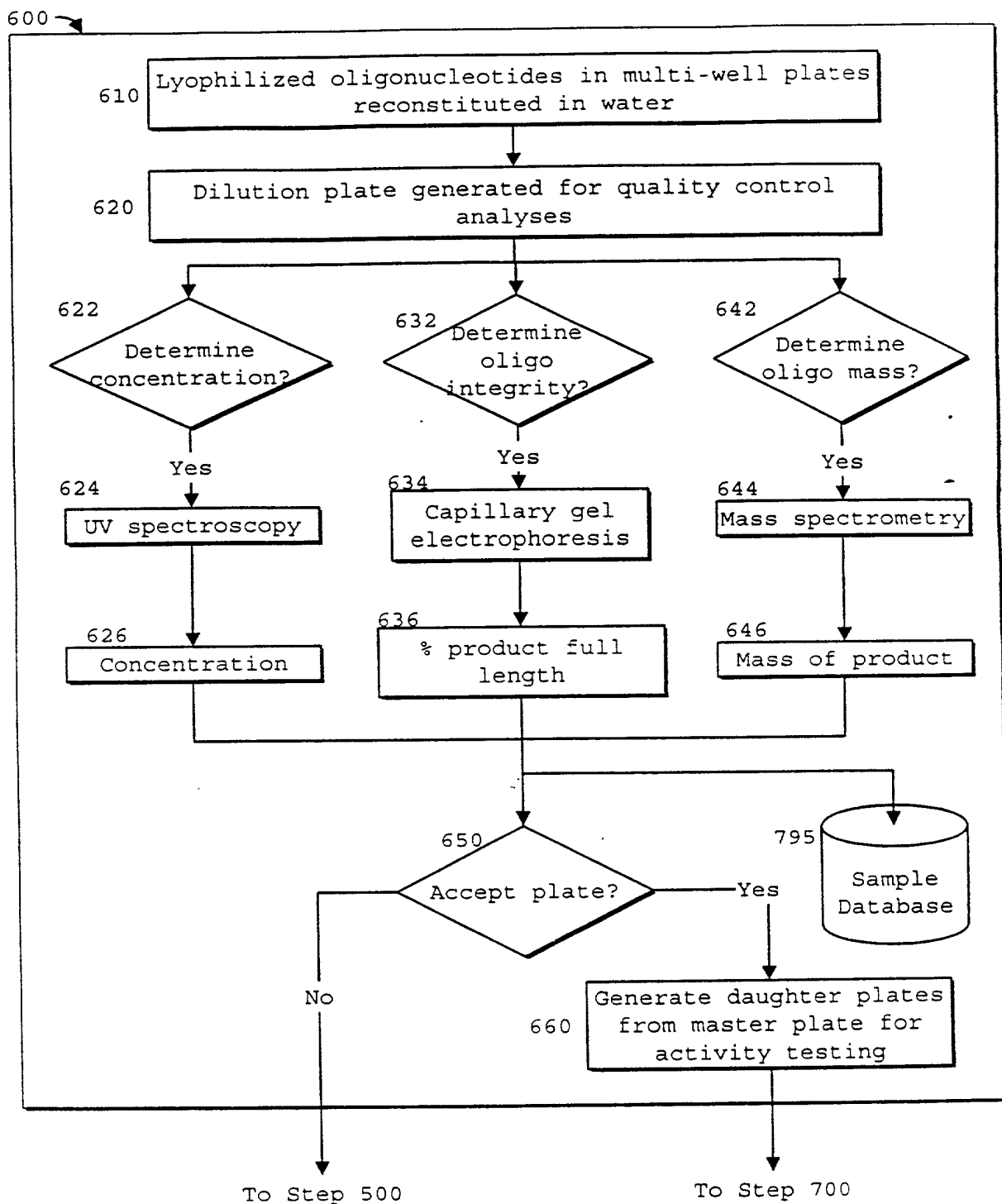


Figure 15

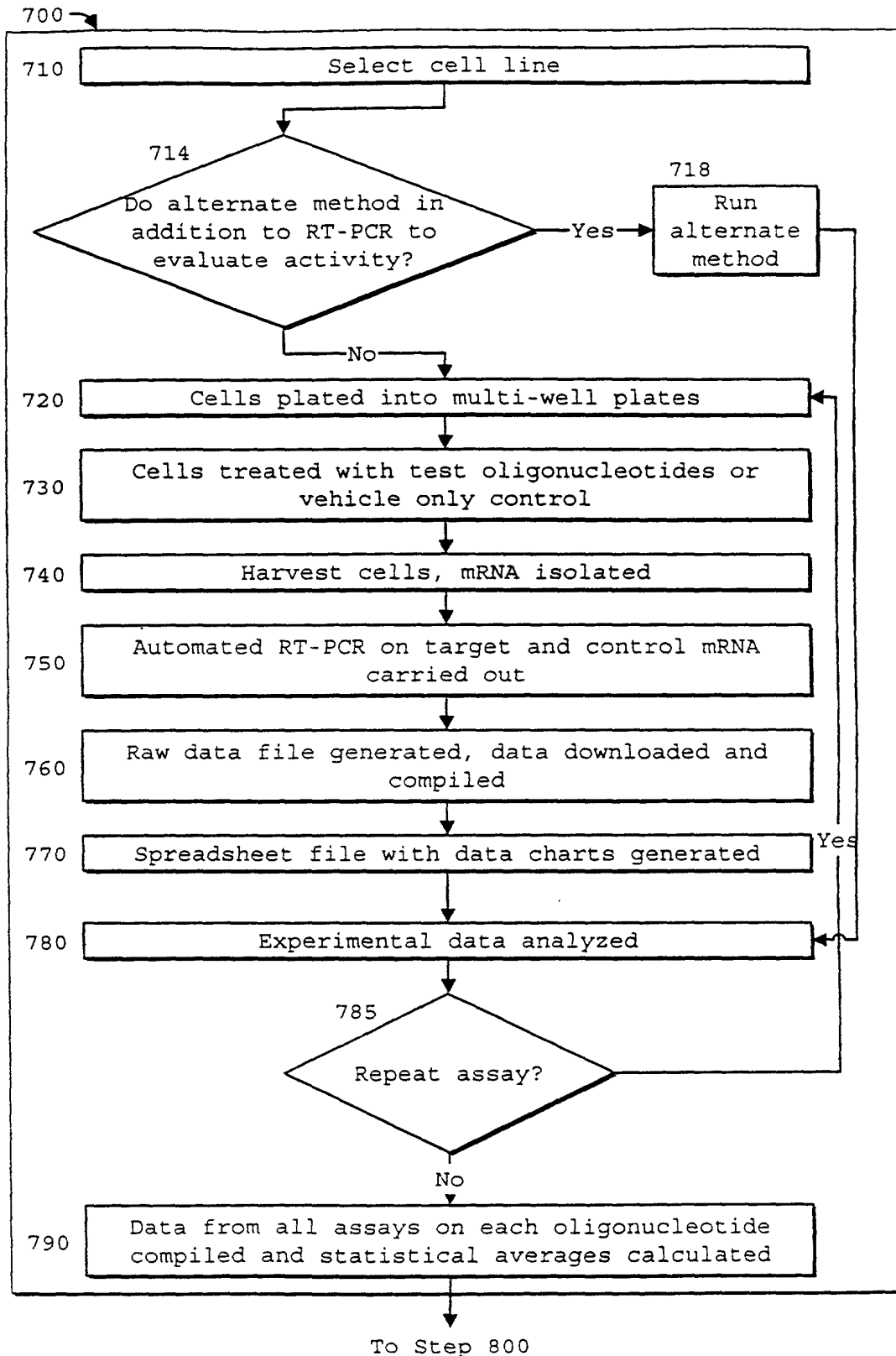


Figure 16

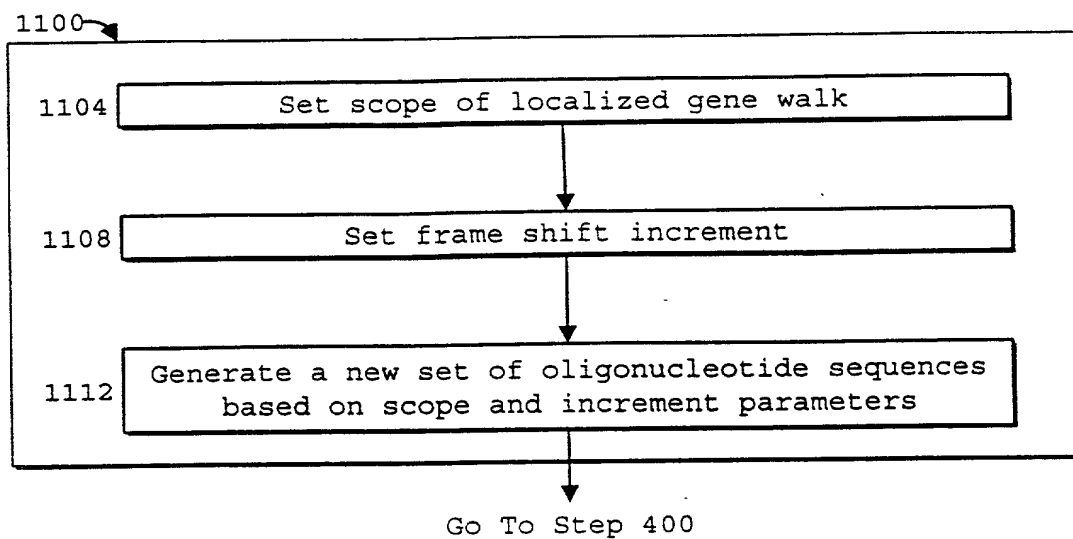


Figure 17

2002 Database Server 2 Processors 1GB RAM 100GB RAID Disk
 2004 Compute Engine 2 Processors 0.5 GB RAM 30GB Disk
 2006 File Server 4 Processors 175GB RAID Disk
 2008 Groupware Server 4 Processors 20GB RAID Disk
 2010 Firewall
 2012 The Internet
 2014 Automated Synthesizer

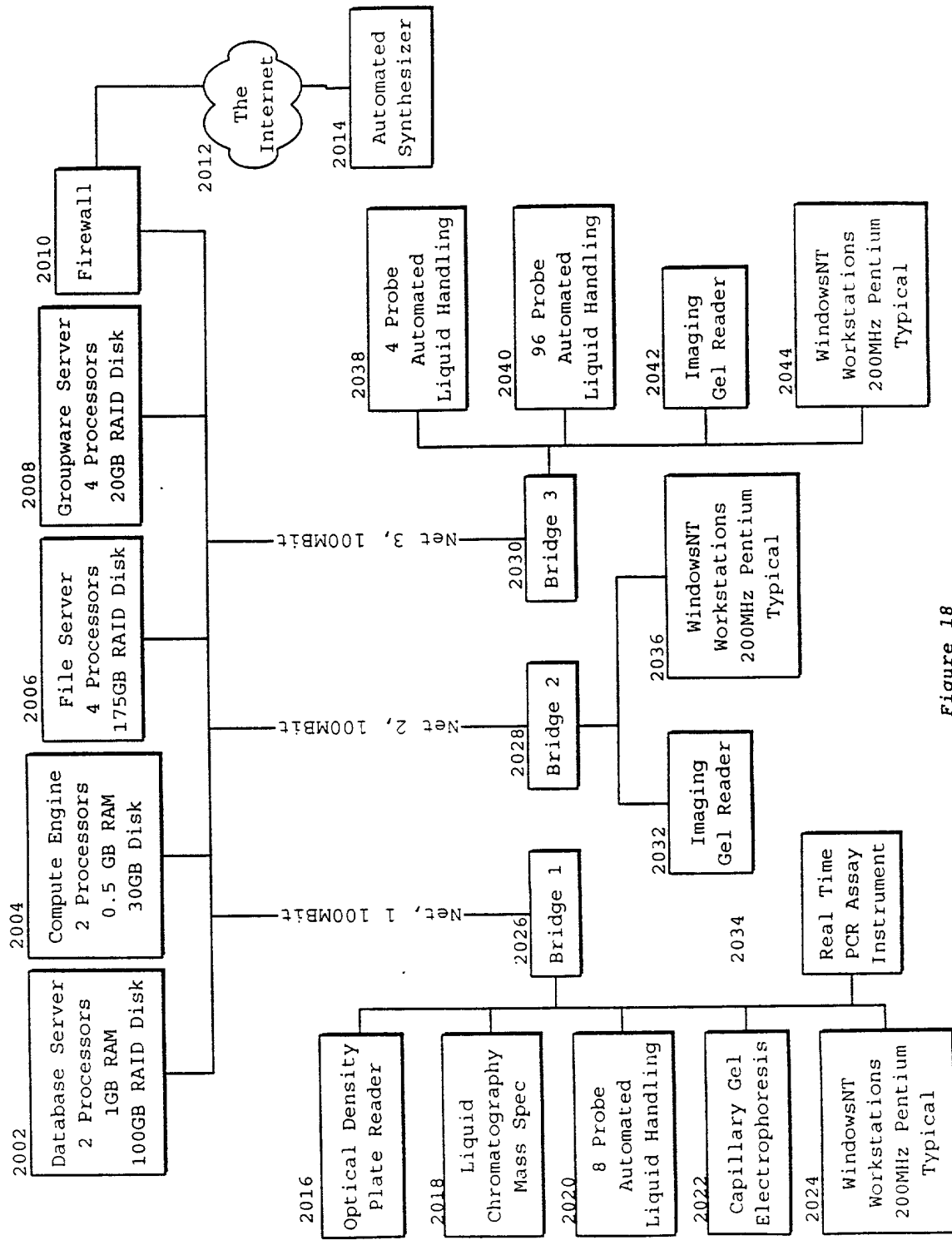


Figure 18

The diagram illustrates the following entities and their attributes:

- OrderEvent**: Date/Time, Person, Status
- Sample**: CompoundID, Lot, Type(Mixture?)
- Oligo**: CompoundID, Chem/Position, Structure, UnitList
- Site**: Target, Position, Length, PickProperties
- Target Database**: Name, Synonyms, Sequence, Components
- Genome Database**: Accession
- Reference**: Journal, Date, Author, Title, Document
- Property Calc Rules**: Name, Instructions
- FoldWalk Properties**: Strategy, ValueDictionary, Target, ID
- Well**: PlateBarcode, Well, Concentration, Sample
- PlateBarcode**: Well, Concentration, Sample
- Data**: Well, Type, Value, Instructions
- Activity**: Sample, Inhibition, Data
- QCData**: Quality, Data, Method, CompoundID
- Oligo Structure**: CompoundID, MolFile
- SampleMoveHistory**: Source, Destination, Date/Time
- Document**: Author, Revision, Date, Contents

Key relationships include:

- OrderEvent** to **Sample** (1:M)
- Sample** to **Oligo** (1:M)
- Oligo** to **Site** (1:M)
- Site** to **Target Database** (1:M)
- Target Database** to **Genome Database** (1:M)
- Genome Database** to **Reference** (1:M)
- Reference** to **Property Calc Rules** (1:M)
- Property Calc Rules** to **FoldWalk Properties** (1:M)
- FoldWalk Properties** to **Well** (1:M)
- Well** to **PlateBarcode** (1:M)
- PlateBarcode** to **Data** (1:M)
- Data** to **Activity** (1:M)
- Activity** to **QCData** (1:M)
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- QCData** to **Oligo Structure**

Figure 19

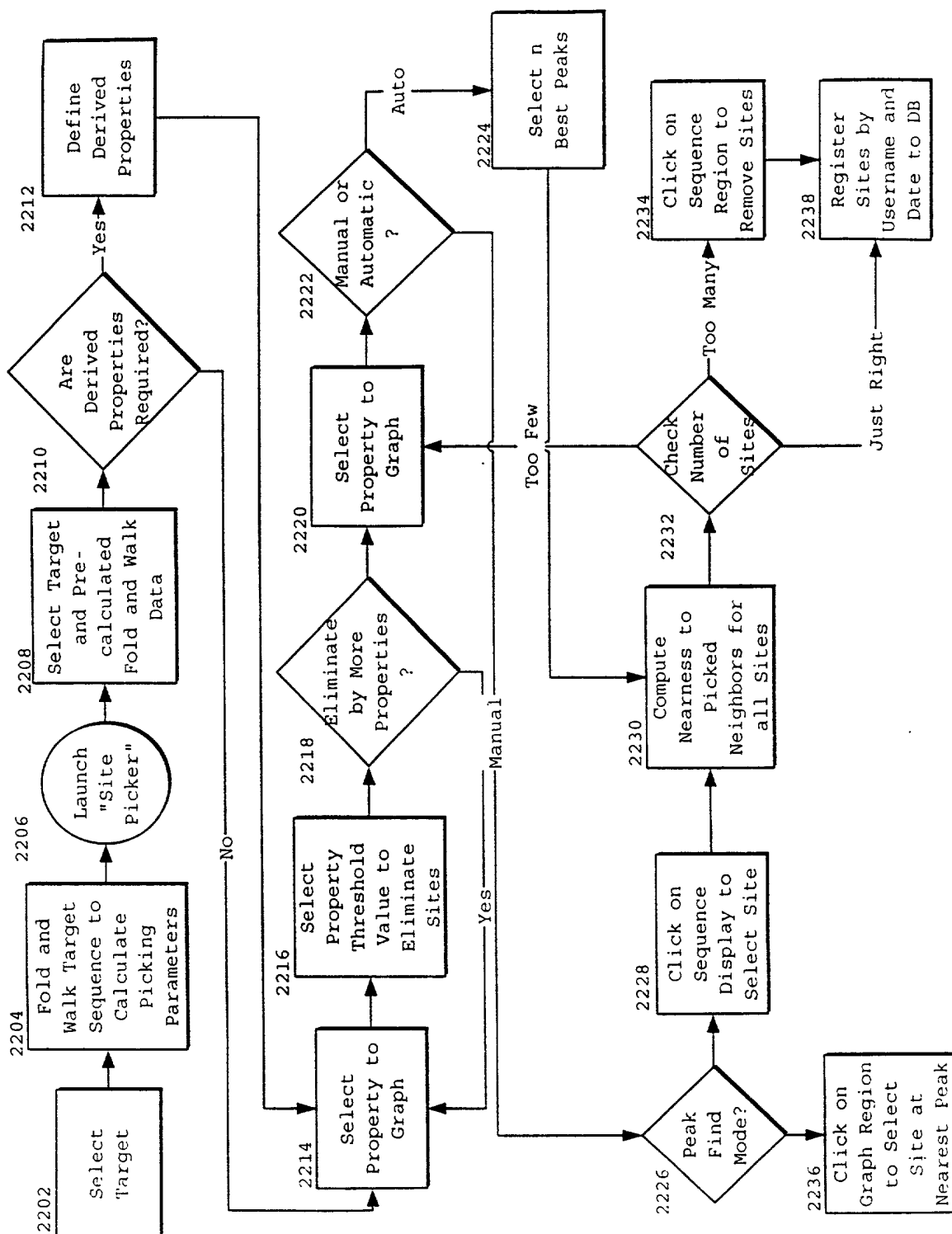


Figure 20

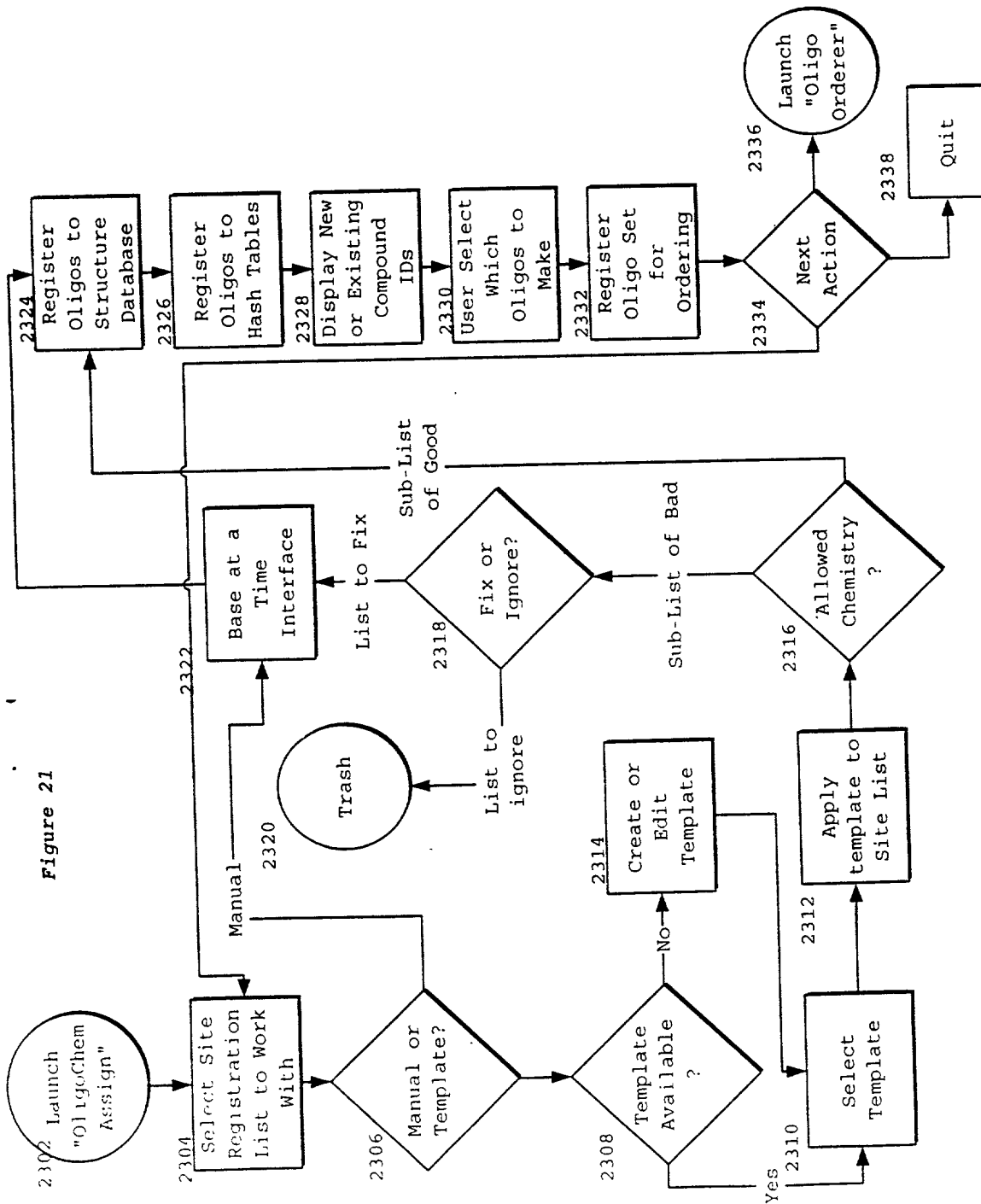


Figure 21

2500

2500 Select desired oligonucleotide sequence length=18

2504 Generate all possible oligonucleotide sequences of length=18 capable of hybridizing to target sequence

2508 Select desired thermodynamic properties

2512 Discard all oligonucleotides of $T_m \leq 40^\circ \text{C}$

2516 Select desired sequence properties

2520 Discard all oligonucleotides containing 4 or more consecutive G's

2524 Discard all oligonucleotides containing 6 or more consecutive repeats of the same base

2528 Of the remaining oligonucleotides, manually select a group that provide a random and uniform distribution along the entire gene target sequence

Figure 22

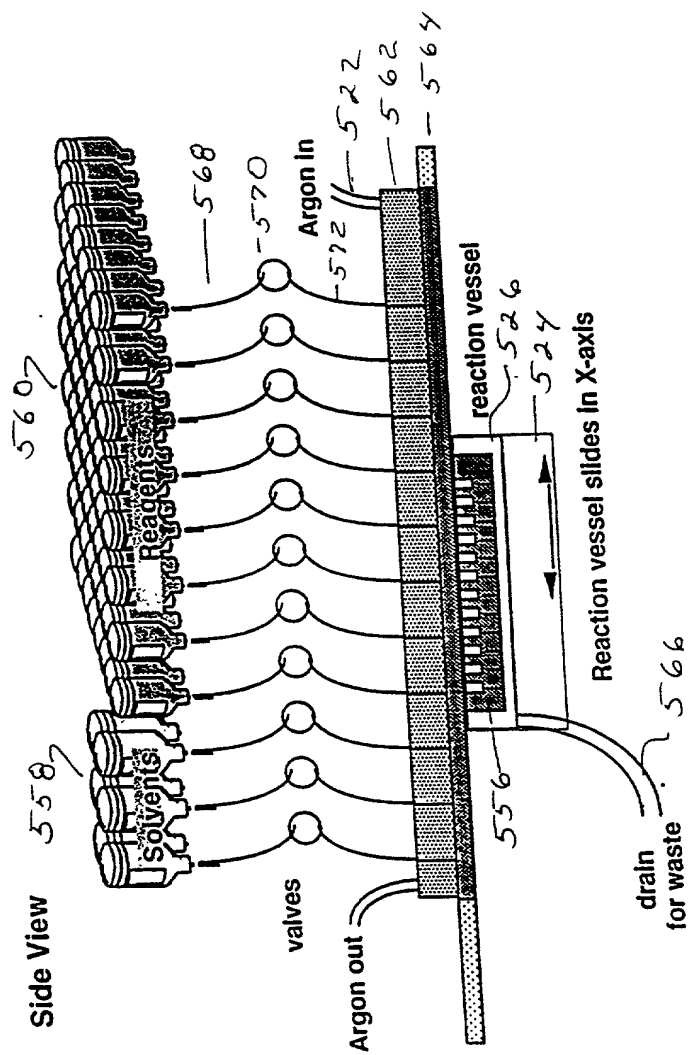


Figure 23

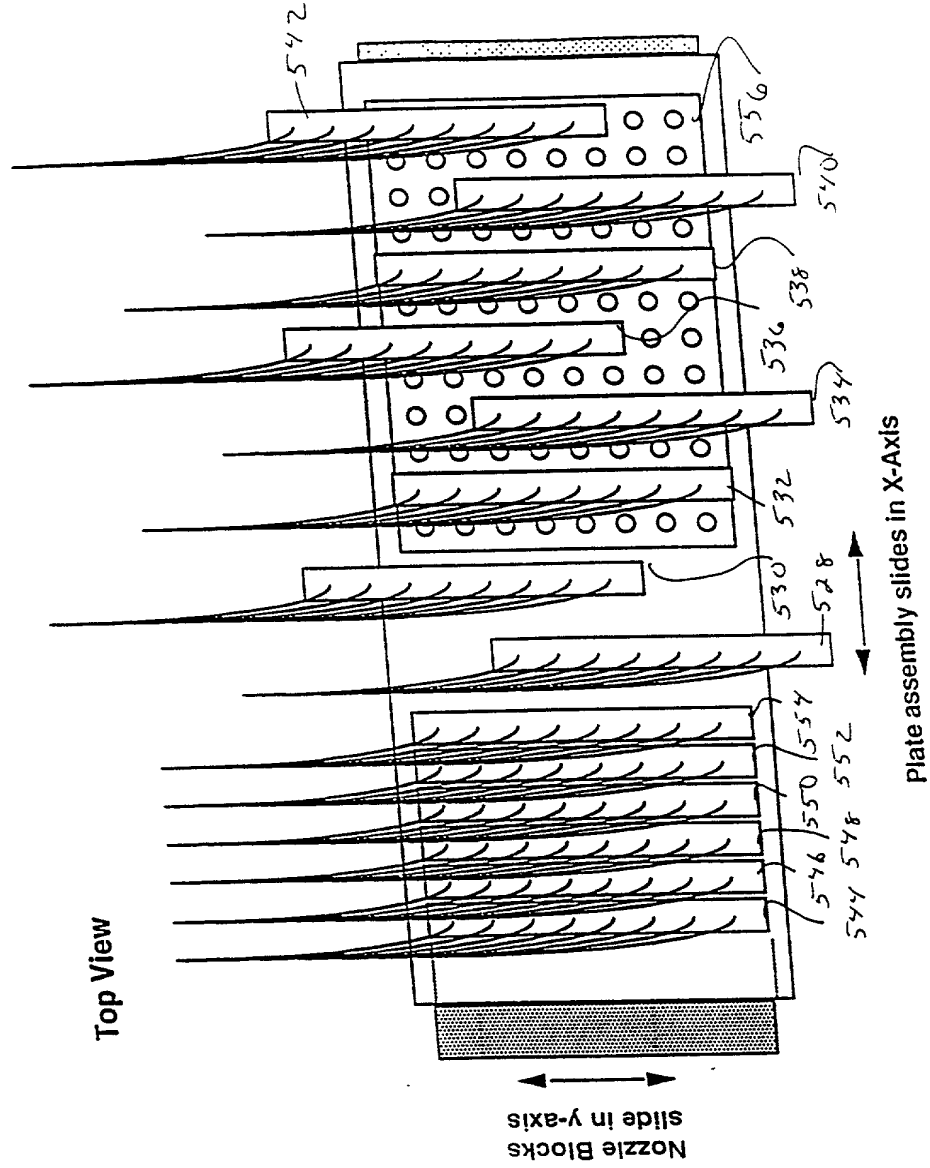


Figure 24

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of:

Lex M. Cowsert, Brenda F. Baker, John
McNeil, Susan M. Freier, Henri M. Sasmor,
Douglas G. Brooks, Cara Ohashi,
Jacqueline R. Wyatt, Alexander H.
Borchers, Timothy A. Vickers

Serial No.: Not Yet Assigned

Group Art Unit: Not Yet Assigned

Filing Date: Herewith

Examiner: Not Yet Assigned

For: IDENTIFICATION OF GENETIC TARGETS FOR MODULATION BY
OLIGONUCLEOTIDES AND GENERATION OF OLIGONUCLEOTIDES FOR
GENE MODULATION

BOX SEQUENCE

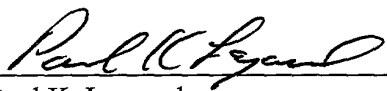
Assistant Commissioner for Patents
Washington DC 20231

STATEMENT TO SUPPORT FILING AND SUBMISSION IN ACCORDANCE
WITH 37 CFR §§ 1.821 THROUGH 1.825

- ☒ I hereby state, in accordance with the requirements of 37 C.F.R. §1.821(f), that the contents of the paper and computer readable copies of the Sequence Listing, submitted in accordance with 37 CFR §1.821(c) and (e), respectively are the same.
- ☐ I hereby state that the submission filed in accordance with 37 CFR §1.821(g) does not include new matter.
- ☐ I hereby state that the submission filed in accordance with 37 CFR §1.821(h) does not include new matter or go beyond the disclosure in the international application as filed.
- ☐ I hereby state that the amendments, made in accordance with 37 CFR §1.825(a), included in the substitute sheet(s) of the Sequence Listing are supported in the application, as filed, at pages _____. I hereby state that the substitute sheet(s) of the Sequence Listing does (do) not include new matter.
- ☐ I hereby state that the substitute copy of the computer readable form, submitted in accordance with 37 CFR §1.825(b), is the same as the amended Sequence Listing.

☐ I hereby state that the substitute copy of the computer readable form, submitted in accordance with 37 CFR §1.825(d), contains identical data to that originally filed.

Date: *April 13, 1999*


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Registration No. 38,534

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COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name; and

I verily believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled: **IDENTIFICATION OF GENETIC TARGETS FOR MODULATION BY OLIGONUCLEOTIDES AND GENERATION OF OLIGONUCLEOTIDES FOR GENE MODULATION** the specification of which:

(XX) is attached hereto.

() was filed on _____ as Application Serial No. _____ and was amended on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information known to be material to the patentability of this application in accordance with 37 CFR § 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. § 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of any application on which priority is claimed:

Country	Number	Date Filed	Priority Claimed
_____	_____	_____	_____
_____	_____	_____	_____
_____	_____	_____	_____

I hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner

provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information known to be material to patentability as defined in 37 CFR § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

Application Serial No.	Filing Date	Status (patented, pending)
<u>09/067,638</u>	<u>April 28, 1998</u>	<u>Pending</u>
<u>60/081,483</u>	<u>April 13, 1998</u>	<u>Pending</u>
_____	_____	_____

I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith: **John W. Caldwell** and **Paul K. Legaard**, Registration Nos. **28,937** and **38,534** of the firm of **WOODCOCK WASHBURN KURTZ MACKIEWICZ & NORRIS LLP**, One Liberty Place - 46th Floor, Philadelphia, Pennsylvania 19103, and **Herb Boswell, Laurel Bernstein and Henry Wu**, Registration Nos. **27,311, 37,280 and 44,412**, of **ISIS Pharmaceuticals**, 2292 Faraday Avenue, Carlsbad, California 92008.

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One Liberty Place - 46th Floor
Philadelphia, PA 19103
Telephone No. **215-568-3100**.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the

United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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	Post Office Address: Carlsbad, California, 92009		
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	Residence: 427 Retaheim Way La Jolla, California 92037	Citizenship: United States	
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	Residence: 2946 Renault Street San Diego, California 92122	Citizenship: United States	
	Post Office Address: San Diego, California 92122		
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	Residence: 2174 Cambridge Avenue Cardiff, California 92007	Citizenship: Canada	
	Post Office Address: Cardiff, California 92007		
8	Full Name: Jacqueline R. Wyatt	Inventor's Signature:	Date
	Residence: 1065 Hymettus Avenue Encinitas, California 92024	Citizenship: United States	
	Post Office Address: Encinitas, California 92024		
9	Full Name: Alexander H. Borchers	Inventor's Signature:	Date
	Residence: 733 Winding Way Encinitas, California 92024	Citizenship: United States	
	Post Office Address: Encinitas, California 92024		
10	Full Name: Timothy A. Vickers	Inventor's Signature:	Date
	Residence: 253 Luiseno Avenue Oceanside, California 92057	Citizenship: United States	
	Post Office Address: Oceanside, California 92057		

SEQUENCE LISTING

<110> Cowsert, Lex M.
Baker, Brenda F.
McNeil, John
Freier, Susan M.
Sasmor, Henri M.
Brooks, Douglas G.
Ohashi, Cara
Wyatt, Jacqueline R.
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Met Ala Ala Ile Arg Lys Lys Leu Val Ile Val
1 5 10

ggg gat ggt gcc tgt ggg aag acc tgc ctc ctc atc gtc ttc agc aag 157
Gly Asp Gly Ala Cys Gly Lys Thr Cys Leu Leu Ile Val Phe Ser Lys
15 20 25

gat cag ttt ccg gag gtc tac gtc cct act gtc ttt gag aac tat att 205
Asp Gln Phe Pro Glu Val Tyr Val Pro Thr Val Phe Glu Asn Tyr Ile
30 35 40

gcg gac att gag gtg gac ggc aag cag gtg gag ctg gct ctg tgg gac 253
Ala Asp Ile Glu Val Asp Gly Lys Gln Val Glu Leu Ala Leu Trp Asp
45 50 55

aca gca ggg cag gaa gac tat gat cga ctg cgg cct ctc tcc tac ccg 301
Thr Ala Gly Gln Glu Asp Tyr Asp Arg Leu Arg Pro Leu Ser Tyr Pro
60 65 70 75

gac act gat gtc atc ctc atg tgc ttc tcc atc gac agc cct gac agc 349
Asp Thr Asp Val Ile Leu Met Cys Phe Ser Ile Asp Ser Pro Asp Ser
80 85 90

ctg gaa aac att cct gag aag tgg acc cca gag gtg aag cac ttc tgc 397
Leu Glu Asn Ile Pro Glu Lys Trp Thr Pro Glu Val Lys His Phe Cys
95 100 105

ccc aac gtg ccc atc atc ctg gtg ggg aat aag aag gac ctg agg caa 445
Pro Asn Val Pro Ile Ile Leu Val Gly Asn Lys Lys Asp Leu Arg Gln
110 115 120

gac gag cac acc agg aga gag ctg gcc aag atg aag cag gag ccc gtt 493
Asp Glu His Thr Arg Arg Glu Leu Ala Lys Met Lys Gln Glu Pro Val
125 130 135

cgg tct gag gaa ggc cgg gac atg gcg aac cgg atc agt gcc ttt ggc 541
Arg Ser Glu Glu Gly Arg Asp Met Ala Asn Arg Ile Ser Ala Phe Gly
140 145 150 155

tac ctt gag tgc tca gcc aag acc aag gag gga gtg cgg gag gtg ttt 589
Tyr Leu Glu Cys Ser Ala Lys Thr Lys Glu Gly Val Arg Glu Val Phe
160 165 170

gag atg gcc act cgg gct ggc ctc cag gtc cgc aag aac aag cgt cgg 637
Glu Met Ala Thr Arg Ala Gly Leu Gln Val Arg Lys Asn Lys Arg Arg
175 180 185

agg ggc tgt ccc att ctc tga gatccccca aagggcctt ttctacatg 688
 Arg Gly Cys Pro Ile Leu
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ccccctcct tcacaggggt acagaaatta tccccctaca accccagcct cctgagggct 748

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 agccatgcac aaaactacct ccctagagaa aggctagtcc cttttctcc ccattcattt 720
 catt atg aac ata gta gaa aac agc ata ttc tta tca aat ttg atg 766
 Met Asn Ile Val Glu Asn Ser Ile Phe Leu Ser Asn Leu Met
 1 5 10
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 Lys Ser Ala Asn Thr Phe Glu Leu Lys Tyr Asp Leu Ser Cys Glu Leu
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 tac cga atg tct acg tat tcc act ttt cct gct ggg gtt cct gtc tca 862
 Tyr Arg Met Ser Thr Tyr Ser Thr Phe Pro Ala Gly Val Pro Val Ser
 35 40 45
 gaa agg agt ctt gct cgt gct ggt ttc tat tac act ggt gtg aat gac 910
 Glu Arg Ser Leu Ala Arg Ala Gly Phe Tyr Tyr Thr Gly Val Asn Asp
 50 55 60
 aag gtc aaa tgc ttc tgt tgt ggc ctg atg ctg gat aac tgg aaa aga 958
 Lys Val Lys Cys Phe Cys Cys Gly Leu Met Leu Asp Asn Trp Lys Arg
 65 70 75
 gga gac agt cct act gaa aag cat aaa aag ttg tat cct agc tgc aga 1006
 Gly Asp Ser Pro Thr Glu Lys His Lys Lys Leu Tyr Pro Ser Cys Arg
 80 85 90
 ttc gtt cag agt cta aat tcc gtt aac aac ttg gaa gct acc tct cag 1054
 Phe Val Gln Ser Leu Asn Ser Val Asn Asn Leu Glu Ala Thr Ser Gln

95 100 105 110
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 Pro Thr Phe Pro Ser Ser Val Thr Asn Ser Thr His Ser Leu Leu Pro
 115 120 125
 ggt aca gaa aac agt gga tat ttc cgt ggc tct tat tca aac tct cca 1150
 Gly Thr Glu Asn Ser Gly Tyr Phe Arg Gly Ser Tyr Ser Asn Ser Pro
 130 135 140
 tca aat cct gta aac tcc aga gca aat caa gat ttt tct gcc ttg atg 1198
 Ser Asn Pro Val Asn Ser Arg Ala Asn Gln Asp Phe Ser Ala Leu Met
 145 150 155
 aga agt tcc tac cac tgt gca atg aat aac gaa aat gcc aga tta ctt 1246
 Arg Ser Ser Tyr His Cys Ala Met Asn Asn Glu Asn Ala Arg Leu Leu
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 aaa gca ggc ttt tac tac ata gga cct gga gac aga gtg gct tgc ttt 1342
 Lys Ala Gly Phe Tyr Tyr Ile Gly Pro Gly Asp Arg Val Ala Cys Phe
 195 200 205
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 Ala Cys Gly Gly Lys Leu Ser Asn Trp Glu Pro Lys Asp Asn Ala Met
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 Ser Glu His Leu Arg His Phe Pro Lys Cys Pro Phe Ile Glu Asn Gln
 225 230 235
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 Leu Gln Asp Thr Ser Arg Tyr Thr Val Ser Asn Leu Ser Met Gln Thr
 240 245 250
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 His Ala Ala Arg Phe Lys Thr Phe Phe Asn Trp Pro Ser Ser Val Leu
 255 260 265 270
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 Val Asn Pro Glu Gln Leu Ala Ser Ala Gly Phe Tyr Tyr Val Gly Asn
 275 280 285

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 Ser Asp Asp Val Lys Cys Phe Cys Cys Asp Gly Gly Leu Arg Cys Trp
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 Glu Ser Gly Asp Asp Pro Trp Val Gln His Ala Lys Trp Phe Pro Arg
 305 310 315

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 Cys Glu Tyr Leu Ile Arg Ile Lys Gly Gln Glu Phe Ile Arg Gln Val
 320 325 330

caa gcc agt tac cct cat cta ctt gaa cag ctg cta tcc aca tca gac 1774
 Gln Ala Ser Tyr Pro His Leu Leu Glu Gln Leu Leu Ser Thr Ser Asp
 335 340 345 350

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 Ser Pro Gly Asp Glu Asn Ala Glu Ser Ser Ile Ile His Phe Glu Pro
 355 360 365

gga gaa gac cat tca gaa gat gca atc atg atg aat act cct gtg att 1870
 Gly Glu Asp His Ser Glu Asp Ala Ile Met Met Asn Thr Pro Val Ile
 370 375 380

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 Asn Ala Ala Val Glu Met Gly Phe Ser Arg Ser Leu Val Lys Gln Thr
 385 390 395

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 415 420 425 430

gag aga gaa aga gca act gag gaa aaa gaa tca aat gat tta tta tta 2062
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 435 440 445

atc cgg aag aat aga atg gca ctt ttt caa cat ttg act tgt gta att 2110
 Ile Arg Lys Asn Arg Met Ala Leu Phe Gln His Leu Thr Cys Val Ile
 450 455 460

cca atc ctg gat agt cta cta act gcc gga att att aat gaa caa gaa 2158
 Pro Ile Leu Asp Ser Leu Leu Thr Ala Gly Ile Ile Asn Glu Gln Glu

465 470 475

cat gat gtt att aaa cag aag aca cag acg tct tta caa gca aga gaa 2206
His Asp Val Ile Lys Gln Lys Thr Gln Thr Ser Leu Gln Ala Arg Glu
480 485 490

ctg att gat acg att tta gta aaa gga aat att gca gcc act gta ttc 2254
Leu Ile Asp Thr Ile Leu Val Lys Gly Asn Ile Ala Ala Thr Val Phe
495 500 505 510

aga aac tct ctg caa gaa gct gaa gct gtg tta tat gag cat tta ttt 2302
Arg Asn Ser Leu Gln Glu Ala Glu Ala Val Leu Tyr Glu His Leu Phe
515 520 525

gtg caa cag gac ata aaa tat att ccc aca gaa gat gtt tca gat cta 2350
Val Gln Gln Asp Ile Lys Tyr Ile Pro Thr Glu Asp Val Ser Asp Leu
530 535 540

cca gtg gaa gaa caa ttg cgg aga cta caa gaa gaa aga aca tgt aaa 2398
Pro Val Glu Glu Gln Leu Arg Arg Leu Gln Glu Glu Arg Thr Cys Lys
545 550 555

gtg tgt atg gac aaa gaa gtg tcc ata gtg ttt att cct tgt ggt cat 2446
Val Cys Met Asp Lys Glu Val Ser Ile Val Phe Ile Pro Cys Gly His
560 565 570

cta gta gta tgc aaa gat tgt gct cct tct tta aga aag tgt cct att 2494
Leu Val Val Cys Lys Asp Cys Ala Pro Ser Leu Arg Lys Cys Pro Ile
575 580 585 590

tgt agg agt aca atc aag ggt aca gtt cgt aca ttt ctt tca tga 2539
Cys Arg Ser Thr Ile Lys Gly Thr Val Arg Thr Phe Leu Ser
595 600 605

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Met Asp Pro Ser Val Thr Leu Trp Gln Phe Leu

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 Gly Pro Lys Glu Glu Leu Glu Val Ala Gly Glu Arg Gly Phe Val Pro
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 Glu Thr Thr Lys Ala Glu Pro Glu Val Pro Pro Gln Glu Gly Val Pro
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 Pro Arg Asp Leu Glu Leu Pro Leu Ser Pro Ser Leu Leu Gly Gly Pro
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aag cgc gac gcc cgg cgc gag ctc aag ctg ctg ctg ctc ggc acg ggc 144
 Lys Arg Asp Ala Arg Arg Glu Leu Lys Leu Leu Leu Gly Thr Gly
 35 40 45

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Gln Asp Val Leu Arg Val Arg Val Pro Thr Thr Gly Ile Ile Glu Tyr
180 185 190

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Pro Phe Asp Leu Glu Asn Ile Ile Phe Arg Met Val Asp Val Gly Gly
195 200 205

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Glu Ser Asp Asn Glu Asn Arg Met Glu Glu Ser Lys Ala Leu Phe Arg
245 250 255

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Ala Arg Glu Phe Ile Pro Lys Met Phe Val Asp Leu Asn Pro Asp Ser
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Asp Lys Ile Ile Tyr Ser His Phe Thr Cys Ala Thr Asp Thr Glu Asn

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